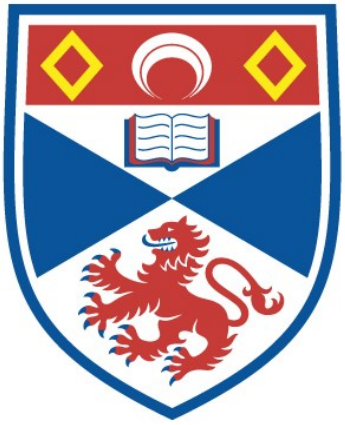
THE MECHANISM OF THE GLUCOSONE INHIBITION OF YEAST FERMENTATION

Ivor L. S. Mitchell

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE MECHANISM OF THE GLUCOSONE INHIBITION

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by

IVOR L. S. MITCHELL, B.Sc.

A Thesis presented to the

University of St Andrews

for the

DEGREE of DOCTOR of PHILOSOPHY.

1954

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a Higher Degree.

The research was carried out in the Department of Physiology and Biochemistry in the United College of St Salvator and St Leonard, St Andrews, under the direction of Stephen Bayne, B.Sc., M.B., Ch.B.

I. L. S. Mitchell.

CERTIFICATE

I hereby certify that IVOR LEWIS STEVENSON MITCHELL has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance 16 (St Andrews), and is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

Stephen Bayne.

CAREER

I first matriculated in the University of St Andrews in October, 1943, before attending a Royal Air Force Short Course from October, 1943 until March, 1944. On returning to the University in October, 1947, I followed a course of study leading to graduation in Science (Biochemistry and Chemistry) in June, 1950. I was awarded 2nd Class Honours in Biochemistry in October, 1951.

I was accepted as a research student in October, 1951 and received a Maintenance Allowance from the Department of Scientific and Industrial Research from that date until June, 1954.

I. L. S. Mitchell.

PREFACE

Glucosone was first prepared by Fischer (1888) who observed that it was not fermented by brewers' yeast. Little biological significance was attached to the compound until Thannhauser and Jenke (1926) reported that glucosone was utilised by diabetics and Hynd (1927a) demonstrated a "glucosone effect" in animals. These investigators regarded glucosone as having an intermediary role in carbohydrate metabolism. More recently Bayne (1945) found that glucosone inhibited the fermentation of glucose by yeast, and further experiments (Mitchell, 1951) showed the the inhibitory effect was specific for the D-isomer, and possibly competitive in nature.

As animal and yeast glycolysis differs mainly in the end-products produced, it was considered that knowledge of the nature of the inhibitory effect on glucose fermentation would give some indication of the mechanism of the "glucosone effect" in animals. The object of this research, therefore, was to investigate the mechanism by which glucosone exerted its inhibitory effect on yeast fermentation.

The results obtained in the fermentation experiments are reported and discussed in Part I. of this thesis. Part II. is a report of experiments carried out with partially

purified hexokinase and a discussion of the results obtained in relation to those reported in Part I. The materials, analytical methods and chromatographic techniques used, are detailed in Part III. The Appendix contains all the data from which the graphs and tables, shown in the thesis, have been compounded.

The author is greatly indebted to Professor A. E. Ritchie for advice and encouragement, to Dr S. Bayne for supervision, to Dr J. A. Fewster for discussions on chemical problems, to Mr J. H. Johnstone for discussions on the purification of hexokinase, and to Mr J. Doyle for proof reading of the manuscript.

The United College,
St Andrews.

June, 1954.

NOTE

Throughout this thesis the D-isomers of the carbohydrates are not specified as such, except in those sections where both D- and L- forms are discussed.

In addition, the following abbreviations are made:-

ATP, adenosine triphosphate.

DPN, diphosphopyridine nucleotide.

TPN, triphosphopyridine nucleotide.

Melting points reported in Part III. are uncorrected.

THE MECHANISM OF THE GLUCOSONE INHIBITION of YEAST FERMENTATION.

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PART I.

INHIBITION OF ANAEROBIC YEAST FERMENTATION

CONTENTS

PART I.

1. AN EXAMINATION OF THE MECHANISM BY WHICH CERTAIN INHIBITORS EXERT THEIR EFFECT ON YEAST FERMENTATION.

1.1. Introduction.

The studies made by Pasteur in the nineteenth century on the metabolism of micro-organisms and yeast, led to his famous statement, "La fermentation est la vie sans air". The demonstration of this statement, which means, in modern terms, that anaerobic fermentation provides energy for the life and growth of the organism, has proved to be a fascinating biochemical study. The transformations occurring in the carbohydrate molecule during the production of this necessary vital energy are now fully understood, the formation of phosphorylated metabolites being the keystone of the schema.

It was not until the end of the 19th century, when Buchner (1897) separated an enzymatic system from yeast which converted sugar into alcohol and carbon dioxide, that any progress was made in the elucidation of the nature of carbohydrate breakdown. The importance of inorganic phosphate was first recognised by Wroblewski (1901) who found that addition of sodium phosphate increased the rate of fermentation of yeast juice. Later, Iwanow (1905), observed

that living yeast could convert inorganic phosphate into organic phosphate. In the same year the fundamental observation of the importance of phosphate in alcoholic fermentation was made by Harden and Young (1905, 1906). In a series of experiments they found that there was an increase in the rate of fermentation of à yeast extract supplemented by added phosphate, when boiled yeast juice was added, indicating that a definite chemical reaction took place between the sugar present and the phosphate added. They also demonstrated a formation of hexosediphosphate and isolated it from fermenting press yeast juice.

Added impetus was given to the growing interest in the mechanism of carbohydrate breakdown when von Lebedew (1911) published a method for a preparation of a maceration juice from brewers' yeast, thus providing an easier method for obtaining an actively fermenting extract. Various esters were isolated from pressjuice and maceration juice fermentations during the next two decades and many observations made of the chemical changes which occurred during glucose breakdown, but it was not until the period 1933-39 that the changes occurring in the hexose molecule during anaerobic fermentation became almost completely understood.

A fuller knowledge of the enzymes concerned in glycolysis has followed with continuing work on the purification of these enzymes, many having been crystallised. The study of the action of inhibitory substances on

fermentation using whole yeast cells, yeast extracts and purified enzymes, has furthered our knowledge in this field, until, at the present time, there is a universally accepted scheme of anaerobic fermentation.

The effects produced by some inhibitors of alcoholic fermentation, and the mechanisms by which these inhibitions are brought about will be discussed here, to determine whether or not any of these mechanisms can account for the inhibitory effects produced by glucosone.

1.2. Inhibition by Cations.

The inhibitory effects of heavy metals on enzymes are well known, and are for the most part non-specific. These effects are exerted at many points in the fermentation sequence as the metals cause precipitation and denaturation of proteins, and are also able to bind thiol groups.

Booy (1940) in a fairly complete investigation of the action of various metals on yeast fermentation found that Al, Be, Cd, Ce, Co, Fe, La, Ma, Ni, Te, Pb, and Zn, all reduced fermentation rate at concentrations below 1.0N, and at higher concentrations all inhibited fermentation completely. The alkali metals and alkaline earths, on the other hand, either stimulated fermentation, or had no effect in concentrations up to 1.0N. Sugar breakdown was inhibited by very low concentrations of Hg, Cu, Ag, and uranyl oxide salts, as might be anticipated from the high toxicity of these metals in biological systems. The inhibitory effect of copper was investigated by Owen (1938), who showed that traces of the metal obtained from the apparatus used in large scale fermentations could reduce the yield of the final products.

Invertase, one of the earliest known carbohydrases, was used as a model for studies on enzyme kinetics by many workers. (Henri, 1902; Michaelis and Menten, 1913). Myrback (1926) demonstrated that silver ions inactivated the

enzyme, the degree of the inhibition being a function of the inhibitor concentration. He suggested that the inhibitory effect was due to a competition by the metal ion with the hydrogen ion necessary for enzymic activity. The metals copper, zinc, cadmium and lead behaved in a similar fashion, but the inhibition produced by mercury was dependent on the substrate concentration. He suggested this was due to an attachment of the mercury to the substrate-combining group of the enzyme. The reversible inactivation of invertase by silver and mercury, was confirmed by Jacoby (1927, 1928) who showed that reactivation could be brought about by dialysis of the enzyme metal complex. He also confirmed that the enzyme was protected from the action of heavy metals by its substrate, a phenomenon which has been noted since with other enzymes and inhibitors (Quastel, and Wheatley, 1931; Hopkins, Morgan, and Lutwak-Mann, 1938; Runnström, Gurney, and Sperber, 1941). Zinc and copper were shown to act on purified invertase in a similar fashion (Boeri, 1947).

The extreme sensitivity of purified phosphoglucomutase to zinc, copper, mercuric and silver ions is said to account for the lack of activity of preparations of this enzyme, in the absence of chelating agents (Sutherland, 1949).

The action of heavy metals on aldolase presents an interesting example of the difference between yeast and animal enzymes, for muscle aldolase is partially inhibited

by 2.0 x 10^{-5}M zinc sulphate (Herbert, Gordon, Subramanyan, and Green (1940) whereas yeast aldolase required zinc, iron or cobalt for complete activity (Warburg and Christian, 1943).

Uranium was shown to combine reversibly with proteins by Barron, Muntz, and Gasvoda, (1948) and by Rothstein and Larrabee (1948). Both groups of investigators also showed that it inhibited the yeast fermentation of glucose. Barron et al (1948) showed that the uranium inhibition of fermentation by whole yeast was reversed by the addition of phosphate or bicarbonate, but a similar inhibition of cell-free fermentation could not be reversed in this manner. It was concluded that the inhibition of yeast fermentation was due to an adsorption of the metal on the cell membrane, which rendered the membrane impermeable to glucose. Rothstein and Larrabee (1948) also showed that uranium acts at the surface of the cell to inhibit yeast fermentation, but in a later investigation Rothstein, Meier, and Hurwitz (1951) concluded that the kinetics of the inhibition were definitely those of an enzyme-inhibited reaction. An inhibition of a phosphorylatin enzyme, probably hexokinase, which was located at the cell-surface was postulated. It is difficult to accept these conclusions as Barron et al (1948) have shown that a partially pure hexokinase is not appreciably inhibited by uranium. A possible competitive effect between the cell enzymes and uranium for available ATP is also discounted as Barron et al

(1948) showed that addition of ATP did not release the inhibition produced by uranium.

It has been shown by many investigators, (Meyerhof, 1917; Dickens, 1934, 1935; Elliot and Baker, 1935) that certain dyes will affect fermentation in living cells under both aerobic and anaerobic conditions. Yeast extracts consume only small amounts of oxygen when metabolising glucose, but the addition of certain dyes which can be reduced by the extract and oxidised by molecular oxygen, cause appreciable oxygen consumption. Lipmann (1934) attempted to correlate this oxygen consumption with the inhibition of fermentation which was produced at the same time. He reported, however, that the oxygen consumption was not related to the degree of inhibition and concluded that the deciding factor is the redox potential which the dye-stuff imposes on the fermenting mixture. Michaelis and Smythe (1936) criticised these findings and reported that the commonly used dyes may be classified into three groups. In the first group are those which increase oxygen consumption but do not inhibit fermentation, e.g. gallocyanine, phrenosafranine and neutral red. The second group including pyrocyanine, methylene blue and thionine, inhibits aerobic fermentation by destroying enzymes; the third group inhibits aerobic fermentation by suppressing the formation of hexosediphosphate, and includes rosinduline GG, 1-naphthol-2-sulphonate, indophenol, and brilliant alizarin

blue. These investigators were unable to find any correlation between inhibitory action and redox potential.

The investigations carried out by Quastel and Yates (1936) on the action of acidic and basic dyes on invertase gave rise to the hypothesis that the enzyme acts as a zwitterion whose oppositely charged groups are bridged by sucrose. They found that the inhibition produced by a given concentration of basic dye increased with increase of pH, and that sucrose and glucose protected against basic dyes and sucrose and fructose against acid dyes. It was considered, therefore, that an anion $OH.E^-$ was formed which combined with the glucose moiety and basic dyes, and the cation $H.E^+$ which bound acid dyes and the fructose part of the sucrose molecule.

1.3. Inhibition by Arsenate.

During their early experiments with yeast juices Buchner and Rapp (1897) found that arsenate did not inhibit cell-free fermentation, but occasionally produced a stimulatory effect. The acceleration of yeast fermentation by low concentrations of arsenate was demonstrated more definitely by Harden and Young (1911), and after an appraisal of the results of many investigators, Harden (1932) concluded that the arsenate was esterified with a sugar molecule. Warburg and Christian (1939a, 1939b) showed that 1:3-diphosphoglyceric acid is normally formed in muscle extracts and suggested that 1-arseno-3-phosphoglyceric acid was formed by these extracts in the presence of arsenate. As the rate of fermentation in yeast extracts is dependent to some extent, on the speed of dephosphorylation of 1:3 diphosphoglyceric acid, ADP being the phosphate acceptor, they suggested that the immediate non-catalytic splitting of 1-arseno-3-phosphoglyceric acid produces an acceleration in fermentation rate. Meyerhof and Junowicz-Kocholaty (1942) later showed that the accelerating effect could also be due to an increase in the speed of phosphopyruvic breakdown, caused by the increase in available ADP brought about by the spontaneous decomposition of 1-arseno-3-phosphoglyceric acid.

An inhibitory effect of arsenate on living yeast cells has, however, been reported by Bleyer and Thies (1942)

and Diemair and Schülke (1942). Bleyer and Thies noted that 96 per cent. of the added arsenate is bound by the yeast after the completion of the fermentation, and Diemair and Schülke have shown that the rate of this binding process increases with an increase in fermentation rate. More recent experiments by Reiner (1948) on the effect of arsenate on the adaptive enzymes of yeast have shown that enzyme formation and nitrogen assimilation are inhibited by arsenate. As this inhibition can be reversed by addition of phosphate, he suggested that one of the main functions of arsenate is the dissociation of carbohydrate metabolism from phosphate esterification.

Arsenite, on the other hand, exerts an inhibitory effect on oxidative enzymes, while stimulating the aerobic fermentation of yeast (Pickett and Clifton, 1943). These authors reported that sodium arsenite inhibited the oxidation of both glucose and pyruvate, but showed that the inhibition of oxidation of glucose could not be referred entirely to an inhibition of pyruvic acid oxidase.

1.4. Inhibition by Fluoride.

The inhibitory effect of fluoride on alcoholic fermentation has been recognised for a long time but it was not until Meyerhof and his co-workers undertook a more detailed investigation of individual enzyme systems that the specific nature of the inhibition was realised.

It was shown, in a series of investigations (Lohmann and Meyerhof, 1934; Meyerhof and Kiessling, 1935; Meyerhof and Schulz, 1938), that fluoride acts specifically on enolase, causing an accumulation of 2-phosphoglyceric acid.

As differences were found in the inhibitory effects produced on whole yeast cells, and in yeast extracts, Malm (1940) studied the permeability of yeast cells towards sodium fluoride. He discovered that it diffused into the cell as undissociated hydrogen fluoride, the sodium hydroxide remaining outside the cell raising the pH and preventing the infiltration of more fluoride. The amount of fluoride absorbed was found to be directly proportional to the outside concentration of the inhibitor, so that the effect was not one of simple adsorption. Runnström (1941) then found that the inhibitory effect of fluoride was governed by the time interval between the addition of glucose and fluoride. In addition to the greater inhibitory effect produced in yeast juice fermentation than that produced in whole cell

fermentation, it was found that the addition of phosphate enhanced the inhibitory effect in either of these systems. In a rather more complete investigation Runnström, Gurney, and Sperber (1941) showed that, at low concentrations, fluoride stimulated fermentation when added before glucose, by inhibiting glyconeogenesis. The addition of alcohol, pyruvic acid, trehalose, or glucose prior to the addition of the fluoride was found to give a protective effect, but arabinose did not affect the inhibition. It was considered, therefore, that a "protected unit" was formed on the enzyme, by a combination of a substrate or metabolite with the enzyme proper. The inhibition was found to change from 0 to 100 per cent. within a very narrow range of inhibitor concentration which suggested a narrow locus of action.

The explanation and the mechanism of the fluoride effect was confirmed by the experiments of Warburg and Christian (1942), who isolated and crystallised yeast enolase and showed it to be inhibited by fluoride. They demonstrated that the magnesium ions required by enolase for complete activity could be replaced by zinc or manganese, these findings being confirmed by Utter and Werkman (1942). Warburg and Christian showed that phosphate concentration influenced the inhibition to a large extent, and that the inhibition produced in a given concentration of phosphate and fluoride, increased with an increase of magnesium concentration. They concluded

that fluoride inhibition of enolase is due to the formation of magnesium fluorophosphate which competes with the magnesium of the enzyme-metal complex. An equilibrium equation was formulated:

Mg.fluorophosphate + Mg.enolase ⇌

Mg.fluorophosphoenolase + Mg.salt.

to explain the effect. Warburg and Christian (1942) showed that the fluoride inhibition of carboxylase, which requires Mg for complete activity, did not vary with the phosphate concentration, so that this mechanism of fluoride inhibition may not be a general one.

Although the inhibition of enolase by the mechanism proposed by Warburg and Christian probably accounts for the inhibitory effects of fluoride on alcoholic fermentation, some anomalous results have been obtained using whole yeast cells which cast doubt on the specificity of the reaction.

Nillson, Alm and Burström, (1942) state that manganese chloride will reverse the inhibitory effect of fluoride on alcoholic fermentation whereas magnesium chloride produces no change in the inhibition. This would suggest that Mn does not form an enzyme-metal-fluorophosphate complex although Mn.fluorophosphate itself may be produced.

Runnström and Marcuse (1943) demonstrated that glucose did not exert any protective effect in press juice fermentations, in which the fluoride inhibition is increased

by phosphate but not by potassium. On the other hand, the fluoride inhibition of fermentation by whole yeast cells is enhanced by the addition of potassium and not by phosphate. A considerable increase of inhibition at pH 5 over that obtaining at pH 6 was also noted with fresh whole cells although with dried yeast the inhibition was independent of the pH. From these findings it was concluded that the sensitivity of the cell towards fluoride depends on the activity of metabolic processes in the cell at the moment the fluoride is introduced.

It has been shown by Runnström, Borei, and Sperber (1940) that certain concentrations of fluoride produced an inhibition of fermentation and a stimulation of respiration, whereas at other inhibitor concentrations the reverse effect could be demonstrated. Respiration was inhibited by fluoride using glucose, pyruvate, methylglyoxal, acetaldehyde, ethyl alcohol, acetic acid and lactic acid as substrates. Fluoride also depressed the dehydrogenase activity of a yeast juice using lactic acid or hexosediphosphate plus glucose as substrates. The reduction of methylene blue by this same yeast juice was not influenced by fluoride, however, so it was concluded that fluoride exerts its depressant effect on a stage intermediate between dehydrogenase and the cytochromes. It was shown by Borei (1940) that fluoride did not exert its effect on cytochrome c.

In a more recent investigation Nickerson and Chung (1952) have confirmed that in non-dividing yeast cells metabolising glucose anaerobically, enolase is the site of fluoride action. A consideration of the results of other experiments led to the conclusion that aerobic fermentation is affected at some other point. The fluoride inhibition of growth was reversed by glucose-1-phospate but not by glucose-6-phosphate, which would seem to locate this effect at the phosphoglucomutase stage. Najjar (1948) has shown that the inhibitory effect of fluoride on crystalline muscle phosphoglucomutase is increased on addition of glucose-1-phosphate so that if the yeast and muscle enzymes are similar the fluoride effect cannot occur at the phosphoglucomutase stage during yeast growth. Nickerson and Chung (1952) have suggested that the inhibitory effect might also occur at the synthesis of glucose-1:6-diphosphate, the co-enzyme for the phosphoglucomutase reaction. This would be more feasible in consideration of Najjar's (1948) findings.

1.5. Inhibition by Cyanide.

Warburg and his co-workers have shown that the cyanide inhibition of respiration and of carbon dioxide and nitrogen assimilation, at low inhibitor concentration, is due to the formation of enzyme-metal-inhibitor complexes in a manner similar to that described for enolase. As several of the intermediary reactions of fermentation are catalysed by enzymes which require metals for their activation, it would appear that KCN could exert its effect at many places in the glycolytic chain. Meyerhof and Lohmann (1934) found that aldolase forms triose phosphate from hexose diphosphate even in 0.1M potassium cyanide solution, although Warburg has demonstrated that this enzyme requires Zn^{++}, Fe^{++}, or Co^{++} for complete activation. The mechanism of cyanide inhibition would, therefore, seem to be different from that obtaining with fluoride.

Massart and Dufait (1942) attempted to localise the inhibition by investigating the sensitivity of various reactions in maceration juice towards cyanide. They demonstrated that the enzymes catalysing the phosphorylation of glucose by phosphopyruvic acid were activated by Mg^{++}, Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}, Cd^{++}, and Fe^{++}, in agreement with the finding of Ohlmeyer and Ochoa (1937), and also that these enzymes were inhibited by cyanide. It was concluded that cyanide inhibition was caused by the formation of an enzyme-metal-inhibitor complex.

Meyerhof and Kaplan (1952) later suggested that this inhibition is much more complex than Massart and Dufait realised, and is brought about by a completely different mechanism. They showed that inhibition of alcoholic fermentation could be produced by the formation of parapyruvate, a dimeric condensation product of pyruvate formed in the presence of cyanide, which was first described by Krebs (1932); and also by the formation of a cyanohydrin by combination of glyceraldehyde phosphate and potassium cyanide. The formation of parapyruvate inhibits fermentation by preventing the further metabolism of pyruvate, as the dimer is not metabolised by carboxylase. The formation of the cyanohydrin removes one of the intermediates in the glycolytic sequence, and also prevents the re-oxidation of DPN.H_2. Only indirect evidence was presented for this last type of inhibition, Meyerhof and Kaplan showing that living yeast fermentation was 55 per cent. inhibited by 0.01M KCN, whereas similar effects were obtained with dried yeast only in the presence of added DPN. No difference was found in the amount of DPN.H_2 present in a cyanide inhibited system, from that found in a normal system however.

The formation of parapyruvate and glyceraldehyde phosphate cyanohydrin probably contributes to the overall effect of cyanide on yeast fermentation, but the main inhibitory

effect was shown to be on alcohol dehydrogenase (Meyerhof and Kaplan, 1952). Meyerhof, Ohlmeyer, and Mohle (1938) have shown that DPN, which is a substrate for alcohol dehydrogenase, forms an addition product with cyanide but the formation of this complex was found to have no effect on the activity of yeast dehydrogenase. It was demonstrated that complete inhibition was produced when 0.001M potassium cyanide was mixed with the enzyme prior to the addition of alcohol and DPN, but only 50 per cent. inhibition when the enzyme was added to a mixture of alcohol, DPN and cyanide. The latter conditions correspond more nearly with those obtained in cyanide-treated living yeast, and gives a similar inhibition index. As crystallised alcohol dehydrogenase does not contain any metal activator (Negelein and Wulff, 1937) the inhibition cannot be due to the formation of a heavy metal-enzyme complex. The inhibition was seen to be very similar to that produced by cyanide on xanthine oxidase, a process which is caused by an irreversible change in the enzyme protein, (Ball, 1939).

It is probable, therefore, that part of the inhibition of alcoholic fermentation by cyanide is produced by the formation of a cyanohydrin compound which prevents further metabolism, rather than by a formation of enzyme-metal-cyanide complexes. Inhibition may also occur at the

alcohol dehydrogenase level, as this enzyme is very sensitive towards cyanide, but as the toxic effect seems to be rather non-specific, other glycolytic enzymes may be similarly poisoned.

1.6. Inhibition by Azide.

The effect of sodium azide on the carbohydrate metabolism of living yeast cells has been studied by many investigators, with contradictory results. The available data on the effect of azide on assimilatory processes in micro-organisms have been reviewed by Clifton (1946).

Azide is known to interfere with several aspects of the anaerobic metabolism of yeast. Spiegelman (1947) reported that adaptive enzyme formation was affected, and Reiner and Spiegelman (1947) demonstrated that carbohydrate assimilation was also inhibited. The latter authors confirmed the findings of Winzler (1944) which had indicated that anaerobic assimilation of glucose did not take place in the presence of azide, as it was found that the theoretical amount of carbon dioxide, predicted by the Gay-Lussac equation, was produced in the presence of 1×10^{-3}M sodium azide during glucose fermentation.

It was shown later, by Trevelyan, Gamman, Wiggins, and Harrison (1952) that a considerable amount of glycerol was produced during fermentation in the presence of azide, suggesting that the production of this quantity of carbon dioxide was due to a net breakdown of stored carbohydrate sufficient to compensate for the glycerol production.

The stimulatory effect of 1×10^{-4}M azide was confirmed by Fales (1953) but the dissimilation of the

carbohydrate reserve noted at a concentration of 2.5×10^{-3}M by Trevelyan et al (1952) was not found at concentrations of 1×10^{-3}M.

An analysis of the ability of sodium azide to dissociate anaerobic glycolysis from cellular synthetic activity, carried out by Spiegelman, Kamen, and Sussman (1948), showed that there was an inhibition of the conversion of inorganic to organic phosphate. They suggested that "azide uncouples anaerobic oxidation from synthesis by means of a replacement reaction between azide and the acyl phosphate of diphosphoglycerate while the latter is combined with phosphorase". This suggestion is based on the observation that azide could decrease the sensitivity of fermentation towards iodoacetate, iodoacetamide and p-chloromercuribenzoate, as did arsenate under similar conditions. As arsenate acts on the triose phosphate dehydrogenases system (Warburg and Christian, 1939a) it was assumed that the azide effect was exerted at this point. Experiments with isolated triose phosphate dehydrogenase, designed to show that azide did combine with the enzyme were negative, however, so that azide does not replace phosphate in this reaction. The above hypothesis, proposed because of a certain similarity between the azide and arsenate effects, and an elimination of some immediate alternatives by experiments with isolated systems, would therefore seem to be unproven.

Reiner (1948) has suggested that the action of azide differs from that of arsenate and is unlikely to be due simply to the suppression of high-energy phosphate bond generation.

The action of azide on living yeast can be explained by one of two mechanisms according to Trevelyan et al (1952), who showed that synthesis of glycogen by living yeast occurs when the ratio glucose:glucose-1-phosphate exceeds 0.2. In the first place, the effect might be due to a competition between azide and phosphate for the phosphorylase-glycogen complex, by a mechanism similar to that proposed for the action of arsenate on the phosphorylase of Pseudomonas saccharophila by Doudoroff, Barker, and Hassid (1947). The consequent lowering of glucose-1-phosphate concentration would then reduce glycogen formation in the cell. No evidence was presented for this hypothesis. On the other hand a rise in the ortho-phosphate concentration could equally cause a diminution in glycogen synthesis, and experiments were described to show that such a rise occurs in the presence of sodium azide. No proof of the source of this increase in orthophosphate was supplied, although it has been shown that metaphosphate synthesis in yeast is inhibited by concentrations of azide which do not interfere with fermentation (Wiame, 1949). It is possible that the

orthophosphate is formed at the expense of organic phosphate, which would agree in general with the conclusions of Spiegelman et al (1948).

Rothstein and Burke (1952) have proposed that the action of azide is explicable on the basis of acceleration of dissimilation of carbohydrate reserves, having observed that the negligible endogenous fermentation rate of starved cells was greatly augmented by azide or 2:4-dinitrophenol. An equilibrium is known to exist between glucose-1-phosphate, glucose-6-phosphate and glycogen in yeast extracts, but as some glycogen is stored in living yeast cells some controlling system is postulated which prevents the fermentation of glycogen. Azide and DNP, it was suggested, reversed this "uncoupling" of glycogen from the fermentation cycle, and so caused an increase of fermentation rate. No further evidence was presented for any of these postulated mechanisms, and as Trevelyan et al (1952) have shown, by direct measurement, that there is no increase in the endogenous utilisation of stored carbohydrate, the hypothesis would seem to require confirmation.

Fales (1953), who reported no new experimental results on the azide effect, criticised the conclusions of Rothstein and Burke (1952), and of Trevelyan et al (1952), and suggested that Spiegelman et al (1948) had provided the best explanation of the phenomenon, should it be limited to

a single site.

There were differences in the experimental results of the various investigations discussed here but as different yeast strains, suspension fluids and inhibitor concentrations have been used, these differences are to be expected, but may, on the other hand, indicate several loci of action.

1.7. Inhibition by Organic Acids and Related Compounds.

Very little is known concerning the mechanism of the inhibition produced by the small number of organic acids and their derivatives which have been investigated.

1.7.1. Organic acids and aldehydes.

One of the earliest records of acid inhibition of fermentation is the report of Rosenblatt and Rozenband (1909), who stated that 0.5N-acetic acid, 0.17N-benzoic acid and 0.17N-salicylic acid, could arrest fermentation. Owen (1938) has stated that the formic, oxalic, butyric, acetic, and citric acids formed during the fermentation of blackstrap molasses, all have inhibitory effects, but gives no details of the concentrations encountered or of the inhibition produced. Sodium ascorbate has no effect on beer yeast fermentation according to Arloing, Morel, Josseraud, and Perret (1937) but sodium dehydro-ascorbate was shown to have a weak inhibitory effect. The $FeCl_2$, $FeCl_3$, and ferrous complexes of dehydro-ascorbic acid were strong inhibitors, while the ferric complex of dehydro-ascorbic acid had very little inhibitory action.

In a study of the effects of salicylic acid and derivatives on apozymase fermentation of glucose, von Euler and Allström (1943) showed that the inhibition produced by sodium salicylate varied with the inhibitor concentration and also with the concentration of the cozymase present.

It was demonstrated that salicylaldehyde produced a much stronger inhibitory effect, but that acetyl salicylic acid was only slightly inhibitory. Sodium salicylate also inhibited living yeast fermentation and carboxylase activity, although to a much smaller extent than apozymase activity. Green, Needham, and Dewan (1937) reported that the glyceraldehyde dehydrogenase activity of various animal tissues is strongly inhibited by salicylaldehyde, although salicylaldehyde itself is metabolised by this enzyme system.

It would appear possible, therefore, that some of the aldehydes which display inhibitory effects do so by competing with a normal substrate for an appropriate enzyme surface. No evidence has been presented which explains the inhibitory effects of the organic acids.

1.7.2. Organic Halogen Acids.

The halogenated acetic acids have been recognised for a long time, monochloroacetic acid being discovered by Leblanc (1844), the iodo-, and bromo- derivatives being synthesised by Perkin and Duppa (1858), some years later.

The first report of the effect of the halogen acids on fermentation is that of Rosenblatt and Rozenband (1909) describing the complete inhibition of fermentation by various concentrations of the mono-, di-, and trichloroacetic acids. Rather later, Lundsgaard (1930a, 1930b) reported

that alcoholic fermentation of sugar by yeast and yeast extracts, and lactic acid fermentation by muscle extracts were inhibited by concentrations of monoiodoacetic acid as low as 5.0×10^{-5}M. The inhibition was found to be specific for fermentation not affecting respiration or yeast cell growth.

Cayrol (1931) then reported that propionic and higher fatty acids, or the chloro-derivatives of these acids, were much less active than bromoacetic and iodoacetic acids. In a more complete investigation of the effects of these acids, Lundsgaard (1932) also found that monobromoacetic acid had the same effect as the iodo-derivative, but that chloroacetic acid, bromopropionic acid and higher homologues were less active.

During the next year, as a result of experiments with many different compounds, Cayrol (1933) formulated empirical rules which govern the effects produced by these acids. It was found that the inhibitory function depends on the group CH_2XCOOR when X is Br or I, in an acid medium below pH 5, a similar effect being produced by iodo- and bromo- substituted acids or the substituted esters of these acids. Inhibition is produced by the esters when the alcohol radical has from one to eight carbon atoms, and is an aliphatic alcohol, a cyclic alcohol or a polyalcohol, but the inhibitory effect is reduced in direct proportion to the size of the aliphatic hydrocarbon substituent.

Some knowledge of the mechanism of the inhibitory effects of the halogenated acids was obtained when Dickens (1933) showed that iodoacetic acid reacted stoichoimetrically with sulphydryl compounds such as glutathione or cysteine, and suggested that this action provided an explanation for the inhibition of glyoxalase by these acids. Independently, Rapkine (1933), reached these same conclusions, showing that iodoacetate reacted with -SH groups but not -SS- groups in egg albumin. The locus of action of iodoacetic acid on yeast fermentation was considered to be triosephosphate dehydrogenase by Green, Needham, and Dewan (1937) and by Rapkine (1938), who showed that the enzyme was one requiring -SH groups for complete activity, being inhibited by iodoacetic acid and iodoacetamide. It was shown by Dixon (1937) however, that although many dehydrogenases were partially inhibited by 1×10^{-2}M iodoacetate, of the twelve dehydrogenases studied(being obtained from yeast, milk, muscle and liver) the alcohol dehydrogenase of yeast was the most sensitive, being inhibited by concentrations of iodoacetate as low as 3×10^{-4}M.

Most metabolic studies using the halogenated organic acids have been carried out using iodoacetic acid rather than bromoacetic acid, as the latter is a deliquescent substance, difficult to handle. Iodoacetamide is also widely used, although the inhibitory effect is not as great as that of the acid.

Smythe (1936) showed that iodoacetamide was required in higher concentration than iodoacetic acid for the comparable inhibition of fermentation by whole yeast cells and yeast extracts. These results demonstrate incidentally, that the inhibitory effect is not caused by permeability changes at the cell surface.

Many investigators have noted that the degree of inhibition of whole cell fermentation by iodoacetate is dependent on pH. Ehrenfest (1932) reported that fermentation is completely inhibited by 3.6×10^{-4}M iodoacetate at pH 4.6, but the inhibition decreases with increase of pH, until at pH 7.0 the inhibitor produces no effect. Cayrol (1933) noted that the inhibitory effect of iodoacetate was maximum at pH 4.0 falling to zero at pH 6.5. In a more complete study of this phenomenon, Aldous (1948) showed that the irreversible toxic effect produced by iodoacetate below pH 5.0 is directly proportional to the amount of undissociated acid present. It was demonstrated that above pH 5.0 the inhibition produced could be reversed, confirming the finding of Kinsey and Grant (1947), who reported that the inhibition produced by iodoacetate was completely reversed in 20 hours by re-suspending the inhibited cells in fresh suspension medium at pH 5.5.

Two other halogenated organic acids, p-chloromercuribenzoic acid and o-iodosobenzoic acid, were introduced as -SH inhibitors by Hellerman, Chinard, and Ramsdell

(1941), and have been widely used in this role. Both produce a reversible inhibition of fermentation.

It is pertinent at this point to consider the work of Barron and Singer (1945) on sulphydryl reagents. These authors point out the danger of using single reagents in attempts to identify -SH requiring enzymes, as these groups show different degrees of affinity for oxidising agents, alkylating agents, and mercaptide-forming compounds. Barron and Singer have suggested that the -SH requirement for enzyme activity should be investigated using iodo-acetamide, p-chloromercuribenzoate, and trivalent arsenicals. As the last two combine reversibly, cysteine and glutathione can be used to reactivate the inhibited enzyme. Much of the earlier work on the determination of the -SH requirement of enzymes was carried out using only one inhibitor, so that it is possible that some glycolytic enzymes other than those which have been tested have a sulphydryl requirement which has not been demonstrated.

The inhibitory effect of the halogen-substituted acids and their derivatives on the fermentation of glucose by living yeast may occur, therefore, at many metabolic levels. The investigations of Dixon (1940) however, make it very probable that alcohol dehydrogenase is the main site of the inhibition produced.

1.8. Inhibition by Other Organic Compounds.

Various other inhibitors of yeast fermentation have been described although very little is known concerning the mechanism of their inhibitory effects.

In an early study on the effects of narcotics on living yeast fermentation Dorner (1912) showed that ethyl-, propyl-, isobutyl-, and phenyl-urethane were all inhibitory, as were phenyl urea, valeronitrile, methyl phenyl ketone and amyl alcohol. A more recent investigation by Segal, (1938) has demonstrated that the inhibitory effect of the higher alcohols on the alcoholic degradation of sugar, increases with molecular weight in an homologous series.

Warburg (1914) stated that phenylurethane, n-octyl and capryl alcohols completely inhibited respiration, assimilation and fermentation in living cells but did not inhibit fermentation in extracts. He suggested that the difference was due to the adsorption of enzymes at the interfaces of the living cell. The narcotic concentration would be much higher at these areas than in the remainder of the cell. Meyerhof and Wilson (1948) demonstrated that brain apyrase was not inhibited by narcotics, although it was shown to be adsorbed onto the cell structure in brain extracts. On the other hand, hexokinase was inhibited by narcotic substances, although in solution in these extracts. It would appear, therefore, that Warburg's hypothesis is not a general one.

More recently Schacter (1953) has stated that the inhibitory effects of $\alpha\alpha'$-diethyl-4:4'-stilbenediol on glucose fermentation are due to an adsorption of the molecule on the yeast cell surface, thereby preventing sugar assimilation.

Some isolated enzyme systems can be inhibited by organic substances which effectively remove a necessary metal co-enzyme by the formation of metal-complexes. Zuckerkandl, Fleischmann, and Drucker (1934) demonstrated that $\alpha\alpha'$-phenantholine inhibits glycolysis in animal tissues and alcoholic fermentation in yeast extracts, by combining with Fe^{++} added as an activator. Meyerhof (1951) has also reported that cysteine, $\alpha\alpha'$-dipyridyl and $\alpha\alpha'$-phenanthroline will inhibit yeast aldolase by combining with the Zn, Fe^{++}, or Co necessary for its action. There is no report of any inhibitory action of these substances on whole cell fermentation.

1.9. Inhibition by Substrate Analogues.

Several inhibitors of alcoholic fermentation have been described which seem to exert their effect by competing with the normal substrates of glycolytic enzymes.

1.9.1. L-Glyceraldehyde.

The inhibition of anaerobic glucose breakdown in rat sarcoma by D-L-glyceraldehyde was discovered by Mendel (1929) and later Mendel, Bauch, and Strelitz (1931) found that the inhibition was reduced by the addition of small amounts of pyruvate. The inhibitory effect was confirmed by Ashford (1934) and Holmes (1934) using brain tissue, and Needham and Nowinski (1937) using chick embryos.

Adler, Calvet, and Günther (1937) showed that D-L-glyceraldehyde also inhibited the fermentation of glucose by washed, dried yeast, in addition to the glycolysis of brain extracts, but that hexose diphosphate breakdown was not affected. The inhibition of dried yeast fermentation was confirmed by Boyland and Boyland (1938) who reported that the glycolysis of starch and glycogen in muscle extracts was also inhibited by D-L-glyceraldehyde. The glycolysis of hexose monophosphate and hexose diphosphate by muscle extracts was not affected.

The L-isomer of glyceraldehyde was finally shown (by Needham and Lehmann, 1937; and by Mendel, Strelitz, and Mundell, 1938), to be responsible for the inhibitory effect. Baker (1938) also demonstrated the specific nature of the

inhibitory effect by showing that glycolaldehyde, propaldehyde, butaldehyde, and benzaldehyde were not inhibitory towards tumour glycolysis. Dihydroxy acetone was also found to be without effect. The inhibition of glycogen breakdown in muscle extracts reported by Boyland and Boyland (1938), was explained by the results obtained by Lehmann and Needham (1938), who found that the dimeric form of glyceraldehyde present in freshly prepared solutions, inhibited phosphorylase. They were unable to demonstrate any inhibition of muscle glycolysis by L-glyceraldehyde, in the presence of added yeast hexokinase. Sullman (1938), on the other hand, found that the glycolysis of both glycogen and glucose was inhibited by high concentrations of glyceraldehyde, even after addition of hexokinase.

These anomalous results led Stickland (1941) to re-investigate the effect of added yeast hexokinase on glyceraldehyde-inhibited muscle glycolysis. He found that a small excess of hexokinase, above that required to permit rapid utilisation of glucose by muscle extract, was very effective in preventing this inhibition of glycolysis, and suggested that the anomalous results obtained earlier were due to differences in the amounts of hexokinase used by different investigators. Stickland also confirmed that the addition of pyruvate could reverse the inhibition produced by L-glyceraldehyde.

Although these results point to hexokinase as the site of action of L-glyceraldehyde, Adler, Calvet, and Günther (1937) reported that the compound did not inhibit yeast hexokinase, prepared according to Meyerhof (1927). More recently, however, Rudney (1949) has stated that L-glyceraldehyde inhibits the hexokinase activity of beef brain, rat tumour, rat skeletal muscle, and to a smaller extent, inhibits yeast hexokinase prepared according to the method of Meyerhof (1927). Pyruvate did not reverse the inhibitory effect of L-glyceraldehyde on hexokinase activity, but did abolish the inhibition of rat tumour glycolysis.

The mechanism of the inhibitory effect was elucidated, to some extent, by Lardy, Wiebelhaus, and Mann (1950). They showed that a hexose monophosphate, formed when L-glyceraldehyde was incubated with aldolase and hexose diphosphate, inhibited the hexokinase activity of a dialysed, lyophilised brain extract. L-sorbose-1-phosphate, prepared synthetically, produced identical inhibitory effects. As neither the conversion of glucose-6-phosphate to fructose-6-phosphate, nor glucose-6-phosphate to fructose-1:6-diphosphate was inhibited by L-sorbose-1-phosphate, it was concluded that the inhibition was specific for hexokinase.

These results may explain the inhibition produced by L-glyceraldehyde on glycolysis by various tissue extracts, but, as Lardy *et al* (1950) have reported that D-L-glyceraldehyde

and sorbose-1-phosphate are without effect on partially purified yeast hexokinase, some difference between yeast and animal hexokinases must be postulated, or some enzyme(s) other than hexokinase must be inhibited. This view is confirmed by the work of Sols and Crane (1953), who have recently reported that partially purified rat brain hexokinase is inhibited by sorbose-1-phosphate.

The inhibition of tumour and muscle glycolysis by L-glyceraldehyde probably occurs through a similar mechanism but the reported inhibition of yeast fermentation may not be produced in this manner. No evidence has been presented to show how pyruvate can reverse the inhibition of tissue extract glycolysis, but Lardy et al have suggested that by maintaining DPN in an oxidised form, pyruvate may allow D-glyceraldehyde to compete more effectively with the L-isomer for aldolase.

1.9.2. Osones.

D-glucosone, the only osone so far known to exert any significant biological effects, was first isolated by Fischer (1888), who reported that it was not fermented by brewers' yeast. Regarding glucosone as a substituted glyoxal, Levene and Meyer (1915) attempted to oxidise the sugar with kidney tissue having glyoxylase activity, but were unable to detect any chemical change in the osone after this treatment.

A similar investigation was carried out by Efendi and Ryzhova (1939), who incubated glucosone with rabbit liver tissue at pH 6.0. They reported that the osone was slowly converted into gluconic and mannonic acids, but as the acids were identified only by the crystalline form of the phenyl hydrazone prepared from the reaction mixture, little credence can be placed on this finding. The reaction was found to be much slower than the control carried out with methyl glyoxal, and was accelerated by the addition of glutathione. Antoniani (1935) reported that glucosone was not attacked by enzymes present in germinating seeds, either in the presence or the absence of glutathione.

The first indication that glucosone might have some role in carbohydrate metabolism was the report by Thannhauser and Jenke (1926) that diabetics were able to utilise this substance. These authors suggested that the inability to form glucosone, a presumed intermediate in glycolysis, resulted in the condition of diabetes mellitus.

Hynd (1927a) demonstrated, in the next year, that certain toxic effects were produced by glucosone. He reported that the injection of glucosone into normal mice produced an effect very similar to that produced by insulin injections, although maltosone, and lactosone, evoked no response. The products of acid hydrolysis of maltosone and lactosone produced effects identical with those obtained

with glucosone. Hynd concluded that the observed effects were not caused by any toxic impurities.

In a later report Hynd (1927b) showed that, in rats and mice, D-glucosone exerted a protective effect against a subsequent injection of potassium or methyl cyanides, or inhalation of gaseous hydrogen cyanide. Herring and Hynd (1928) confirmed the earlier effects produced in mice, using rabbits, guinea pigs, and cats, and showed that the method of administration had no effect on the symptoms.

The toxic effects produced by the dimeric form of hydroxy methyl glyoxal in mice and rabbits, were reported by Kermack, Lambie, and Slater (1929) to be similar to those obtained by injection with glucosone. It was reported by Sakuma (1931) that methyl glyoxal was three times more toxic than glucosone when injected subcutaneously into mice. The symptoms produced by the injection of glucosone differed from those described by Hynd (1927a), and were similar to those produced by injection of phenyl glyoxal, methyl glyoxal, hydroxy methyl glyoxal or glyoxal.

The role of glucosone as an intermediate in carbohydrate metabolism, proposed by Thannhauser and Jenke (1926), and Hynd (1927a), was refuted by Dixon and Harrison (1932), who reported that no glucosone could be detected in the blood of rabbits in hypoglycaemic convulsions following

administration of insulin, or detected after incubating fructose with liver tissue in the presence of insulin.

Berkeley (1933) on the other hand, showed that an oxidase preparation from the style of a mollusc, Saxidomus giganteus, could produce glucosone using glucose as a substrate. The formation of glucosone was also observed when certain plasmolysed moulds acted upon soluble starch, maltose, sucrose or glucose (Bond, Knight, and Walker, 1937). The method of identification of glucosone as a 2:4-dinitrophenylosazone is however, not specific under these conditions.

In a more recent investigation using $[^{14}C]$-glucose and $[^{14}C]$-glucosone administered orally to rats, Becker and Day (1953) showed that glucosamine can be formed from glucose or glucosone, and that glucose can be converted into glucosone. They suggested that although glucosone may not be a normal intermediate, its formation may be a step in glucosamine synthesis.

The specificity of the "glucosone effect" was confirmed by Bayne (1952) who showed that D- and L-xylosone, D- and L-arabinosone, L-glucosone, D-galactosone, L-gulosone, sedoheptulosone, and 3-O-methyl-D-glucosone, produced no toxic effects in mice, at concentrations four times higher than the lethal concentration of D-glucosone.

D-glucosone was reported to exert a specific inhibitory effect on the fermentation of D-glucose by living

bakers' yeast cells (Mitchell and Bayne, 1952). Indications were obtained which suggested that the inhibition might be competitive in nature, but in a later communication, Johnstone and Mitchell (1953) reported that D-glucosone was a pseudo-irreversible inhibitor, of the type described by Ackermann and Potter (1949). It was also reported that D-glucosone was phosphorylated by a partially purified yeast hexokinase. Preliminary experiments by Doyle (1952) have indicated that the fermentation of D-galactose by galactose-adapted yeast may be inhibited by D-galactosone, and to a greater degree by D-glucosone.

The findings reported here show that D-glucosone exerts a specific inhibitory effect in animal tissues and in whole yeast cells, although there is no evidence to show at what stage in the glycolytic sequence the inhibition does occur.

1.9.3. 2-Deoxy Glucose.

The 2-deoxy pentoses, forming the carbohydrate component of desoxynucleic acids, are the only 2-deoxy sugars known to have any biological importance. 2-Deoxy glucose was first synthesised by Fischer, Bergmann, and Schotte (1920) by dilute acid treatment of glucal. The glucal transformation has also been carried out *in vivo*. Kondo (1924) administered D-glucal to rabbits and isolated 2-deoxy glucose, (identified as the *p*-nitrophenylhydrazone), from the urine, in an amount corresponding to 3 per cent. of

the original glucal, 2-deoxy glucose, administered by mouth, was recovered from the urine in 7 per cent. yield.

Cramer and Woodward (1952) reported that 2-deoxy glucose inhibited glucose fermentation by whole yeast cells, and concluded that, as the inhibition was competitive, the enzyme reaction affected must be one which was rate limiting for the entire fermentation. 2-Deoxy glucose did not affect the initial rate of glucose fermentation by a cell-free extract and did not inhibit the rate of fructose-diphosphate fermentation. The difference in the action of 2-deoxy glucose on glucose fermentation by whole yeast cells and on cell-free extracts was considered to be due to the location of a particular rate-determining enzyme in the cell wall, which was essential for the passage of glucose into the cell under anaerobic conditions. As fructose diphosphate fermentation was not affected, and 2-deoxy glucose was phosphorylated by a partially purified yeast hexokinase, the inhibition of fermentation apparently occurred at the hexokinase level. The aerobic fermentation and growth of yeast cells was also inhibited by 2 deoxy glucose. It was noted later (Woodward, Cramer, and Hudson, 1953) that 2-deoxy glucose was more than three times as inhibitory towards fructose fermentation than glucose fermentation.

1.9.4. Glucosamine.

The phosphorylation of glucosamine was reported by Harpur and Quastel (1949), using brain hexokinase, and using yeast hexokinase, by Grant and Long (1952), and Brown (1951). Brown (1951) isolated an acid-stable monophosphoglucosamine as a product of the reaction between glucosamine and ATP in the presence of crystalline yeast hexokinase. He demonstrated that glucosamine was phosphorylated in position 6. Using partially purified yeast hexokinase Grant and Long (1952) also demonstrated that an acid stable monophosphoglucosamine was formed, but did not determine the position of the phosphate group. More recently Woodward and Hudson (1953) have demonstrated that glucosamine is a competitive inhibitor of anaerobic glucose fermentation. Aerobic fermentation was also inhibited but the inhibition was rather less.

1.9.5. Other Carbohydrate Analogues.

Woodward, Cramer, and Hudson (1953) have reported that some other structural analogues of glucose and fructose produce inhibitory effects on anaerobic and aerobic yeast fermentation. They confirmed the inhibition of anaerobic fermention by glucosone, demonstrated earlier by Mitchell and Bayne (1952), and showed that iso-glucosamine and 2-chloro-2-deoxy-glucose had inhibitory properties. They

indicated that isoglucosamine inhibited fructose fermentation to a greater extent than glucose fermentation and noted that glucosamine inhibited fructose fermentation more than that of glucose. Glucosone was reported to inhibit aerobic fermentation, respiration and growth of yeast cells. A large number of substances were tested for inhibitory properties. Those which were inhibitory differed from glucose or fructose only by a simple change on C2. As 2-deoxy glucose was a stronger inhibitor than D-glucosone, which was in turn more potent than 2-chloro-2-deoxy glucose, it was concluded that the degree of inhibition produced by an analogue was dependent on the substituent at C2.

1.10. Conclusions.

A critical examination of the effects of the selective poisons used in the study of yeast enzyme systems shows that many are non-specific. Despite the fact that more than one enzyme may be affected by a single inhibitor the use of such inhibitors has increased the knowledge of synthetic and degradative processes in the living cell, and also provided information regarding the mechanism of inhibition.

The use of the living yeast cell as a glycolytic system presents further complications, however, as the added inhibitor may exert effects on the cell membrane which influence the entry of normal substrates into the cell.

The inhibitory effects of many solutes can be explained by physical and chemical properties which, in themselves, affect cell membrane permeability and the natural state of cell colloids and proteins. The permeability of the yeast cell towards organic and inorganic substances has not been investigated to any great extent but it is probable that some of the data obtained with plant and animal cells can be applied to this organism.

When the study of cell permeability was made possible by the discovery of plant cell plasmolysis and deplasmolysis (Nägeli, and Cramer, 1855), many botanists became interested in the problems presented. The results of these investigations were placed on a more rational basis when Overton (1895),

after reviewing the existing data, concluded that the rate of penetration of organic substances was determined by their solubility in the lipids assumed to be present in cell membranes.

Overton (1899, 1902) was the first to investigate cell permeability systematically. He used both plant and animal cells, and discovered that there was a general relationship between the ability of various organic compounds to enter living cells and the type of chemical structure which favoured solubility in non-polar solvents. Many deviations from this general statement were found, Wilbrant (1931), for example, demonstrated that non-electrolytes containing amino groups were preferentially absorbed by some plant cells, and weakly acidic non-electrolytes by others. When Collander and Bärland (1933) found that several lipid-insoluble inorganic compounds could enter plant cells, they suggested that the cell membrane consisted of "lipoid" areas and "sieve" areas, either exerting the greater influence, depending on the properties of the permeant.

This general picture of the cell membrane is still sound, although modifications are necessary in order to explain the behaviour of many permeable substances. The physico-chemical properties of the inhibitors discussed earlier (1.2. to 1.9) will now be considered, to show the correlation between these properties and the inhibitions produced.

Brooks (1916), Osterhaut (1936), and Collander (1939), reported that the cell membrane was impermeable to physiological concentrations of inorganic ions, but higher concentrations of metal salts were shown to create abnormal conditions at the cell surface (Osterhaut, 1922). There is little information regarding the nature of these changes, but observations made with erythocyte membranes give some indication of a possible mechanism of action. The red cell membrane is said to consist of lipid and protein layers arranged concentrically, each layer being composed of two double molecular layers of lipids separated by a thin protein layer (Waugh and Schmitt, 1940). Between these concentric layers a great amount of water is held as colloidal lipid. Höber (1946) suggested that inorganic ions produce an effect on red cells by changing the physical state of the membrane colloids, mainly by dehydration. It is possible that yeast cell membranes may be similarly affected.

The metal ions may also cause flocculation of the cell colloids contained inside the cell, the degree of flocculation following the Schulze-Hardy rule (Loeb, 1902), which states that ionic flocculation power increases almost in a geometric progression with increasing valency. These physical changes are probably sufficient to account for the inhibitory effects of metal ions on whole cell yeast fermentation, apart from any additional effects on cell contents.

Acidic dyes are, in general, lipid insoluble (Overton, 1902) but Schulman (1917) showed that many of these dyes are taken up by several types of animal cells. The solubility is probably a function of the size of the non-polar portion of the dye molecule. The basic dyes can diffuse into plant cells quite readily, according to Nirenstein (1920). Once inside the cell, dye stuffs are attached to the cell colloids by a stable electrostatic adsorption which prevents any dissociation and rediffusion out of the cell. It is probable that the disorganisation of the cell colloids thus caused, will interfere with normal glycolysis.

Although strong bases and acids resemble neutral salts in that they are completely ionised and do not pass into cells at low concentrations, weak acids and bases do penetrate cell membranes. Osterhaut (1925) showed that hydrogen sulphide could penetrate the cells of the alga Valonia, only at a pH below which no dissociation of the hydrogen sulpide occurred. The rate of penetration was found to be proportional to the external concentration of the hydrogen sulphide, so that simple diffusion was indicated. From the investigations by Malm (1940) (1.4.) on the diffusion of KF into yeast cells, it would appear that simple diffusion of undissociated HF occurs. Similarly, the entry of iodo-acetate (1.7.2.) into the yeast cell may occur by simple diffusion.

Sodium azide and potassium cyanide probably diffuse directly into the cell as undissociated molecules, for they have small molecular volumes and low dissociation constants. According to Höber (1946), weakly dissociating organic acids enter plant cells by simple diffusion, the larger molecules because of their solubility in the lipid constituents of the membrane, and the acids of small molecular volume by a simple diffusion through the pores of the membrane. Overton (1902) indicated that the aldehydes corresponding to these acids also enter into the cell by simple diffusion. All of these freely diffusible substances will, therefore, exert their inhibitory effect inside the cell, without necessarily having any effect on the cell membrane.

Salicylate and benzoate, which have strong polar properties (Höber and Moore, 1930), will exert definite effects on cell membranes, for Anson (1939) has shown that compounds having such properties cause denaturation of proteins.

Narcotic substances, which include higher alcohols, urethanes, and halogenated hydrocarbons, do not normally react chemically with cell constituents but do exert definite inhibitory effects on yeast fermentation. The mechanism by which this effect is brought about is not yet clear, but two general theories of narcosis have been presented. Independently, Meyer (1899) and Overton (1901) postulated that the same degree of narcosis was produced by any narcotic at a

particular molar concentration in the functional cell lipids.

When Traube (1904) demonstrated that a large number of narcotics were surface active compounds, the second theory of narcosis appeared. This theory postulated that there was a quantitative relationship between narcotic strength and surface activity. Höber (1946) later formulated an adsorption theory, stating that "narcosis occurs when, by raising the concentration of a narcotic, irrespective of its chemical nature, the interfacial tension is lowered to a definite point, due to its adsorption affinity toward the substrate of its inhibitory effect".

Confirmation for such a hypothesis can be obtained from the earlier work of Warburg, (1913, 1914) who observed that the inhibitory effects of narcotics towards enzymes were decreased when the adsorbing extracellular material was removed from enzyme preparations. Warburg (1921) later suggested that narcotics inhibited enzyme activity by an adsorption on enzyme surfaces, thereby preventing access of substrate to the reaction sites. Narcotics may also exert another effect on the cell membrane, for adsorption on the cell wall may obstruct, or block, the pores of the cell membrane, thus preventing the entry of normal substrates.

The mechanisms by which substrate analogues, exert their inhibitory effect has not yet been elucidated. It is probable that either the analogue itself, or some product

of the metabolised analogue, may be the true inhibitory substance. The results of investigations on substrate analogues, reported up to the present time, will be discussed in relation to the results obtained in the present work, in a later part of this thesis (Part I. 2.4.).

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2. THE EFFECTS OF GLUCOSONE ON YEAST FERMENTATION.

2.1. Introduction.

The early work carried out by Brown (1892) and Slater (1906, 1908) on the kinetics of alcoholic fermentation by living yeast, established that the rate of fermentation is approximately constant over a wide range of sugar concentrations. Slater found that the rate of fermentation remains constant during the main part of the fermentation. Although these findings were criticised by Nord and Weichhertz (1929), Hopkins and Roberts (1935) showed that Slater's results were true for yeast concentrations between 5.0-0.1 per cent., and for glucose concentrations between 0.056-1.680M. They showed that within this range of concentrations of yeast and glucose, the enzyme surface was completely saturated with substrate, allowing of a constant velocity of action over a wide substrate concentration range. They stated, that providing certain factors which influence the rate of fermentation of glucose by living yeast are carefully considered (i.e. temperature, pH, and rate of agitation), the kinetics are similar to those of a unimolecular enzyme reaction.

More recently Hurwitz and Rothstein (1951) have applied the Michaelis-Menten equation in describing the effects of uranium on the fermentation of glucose and

fructose. They assumed that under the conditions of their experiments, the enzyme was completely saturated by the substrate; thus the addition of uranium gave rise to a reversible inhibition which was non-competitive with glucose as substrate, and competitive with fructose. Cramer and Woodward (1952) and Woodward and Hudson (1953) have also regarded the fermentation of glucose by whole yeast cells as a unimolecular reaction, and have investigated the competitive effects of 2-deoxy glucose and glucosamine in this system.

It was considered, therefore, that under properly controlled conditions the effects of glucosone on the fermentation of glucose, by whole yeast cells, could be investigated on the assumption that the kinetics are those of a unimolecular reaction.

2.2. Methods.

Suspensions of fresh bakers' yeast (Standard Yeast Co.) were made by weighing out required amounts taken from a freshly cut surface of a yeast cake, into 0.02M potassium dihydrogen phosphate. It was found that the control fermentation carried out on each suspension did not vary significantly for each batch, providing the sample of yeast used was not more than a week old.

Yeast suspensions were also made from commercial dried yeast pellets (DCL) by weighing into 0.02M potassium dihydrogen phosphate, and allowing the mixture to stand for 45 minutes before use. The pellets were kept in a stoppered tin at room temperature, over a period of two years without showing any change in fermentative ability.

The gas output was measured manometrically by the Warburg technique at 30°, the rate of carbon dioxide evolution being taken as a measure of the rate of fermentation. There is no retention of carbon dioxide in solution at the initial pH of 4.8 (= pH of 0.02M potassium dihydrogen phosphate solution), and, as an actively fermenting yeast tends to approach pH 3.5, according to Rothstein and Enns (1946), there can be no carbon dioxide retention after the start of fermentation. In these experiments the final pH was found to be within the range of 3.5-4.0.

The yeast suspension was tipped into the main vessel containing substrate, or substrate plus inhibitor, after 10 minutes equilibration at 30°, in the majority of the experiments described. Other procedures are described in the text.

All the experiments were carried out at 30°, with a total fluid volume of 3.0ml.

In the earlier experiments, when KCN was used as inhibitor of the respiration, the yeast suspension was shaken with cyanide for 30 minutes before use. The final concentration of cyanide in the Warburg flasks was 2.6×10^{-4}M.

Later experiments were carried out in an atmosphere of nitrogen which was introduced prior to the 10 minute equilibration period at 30°.

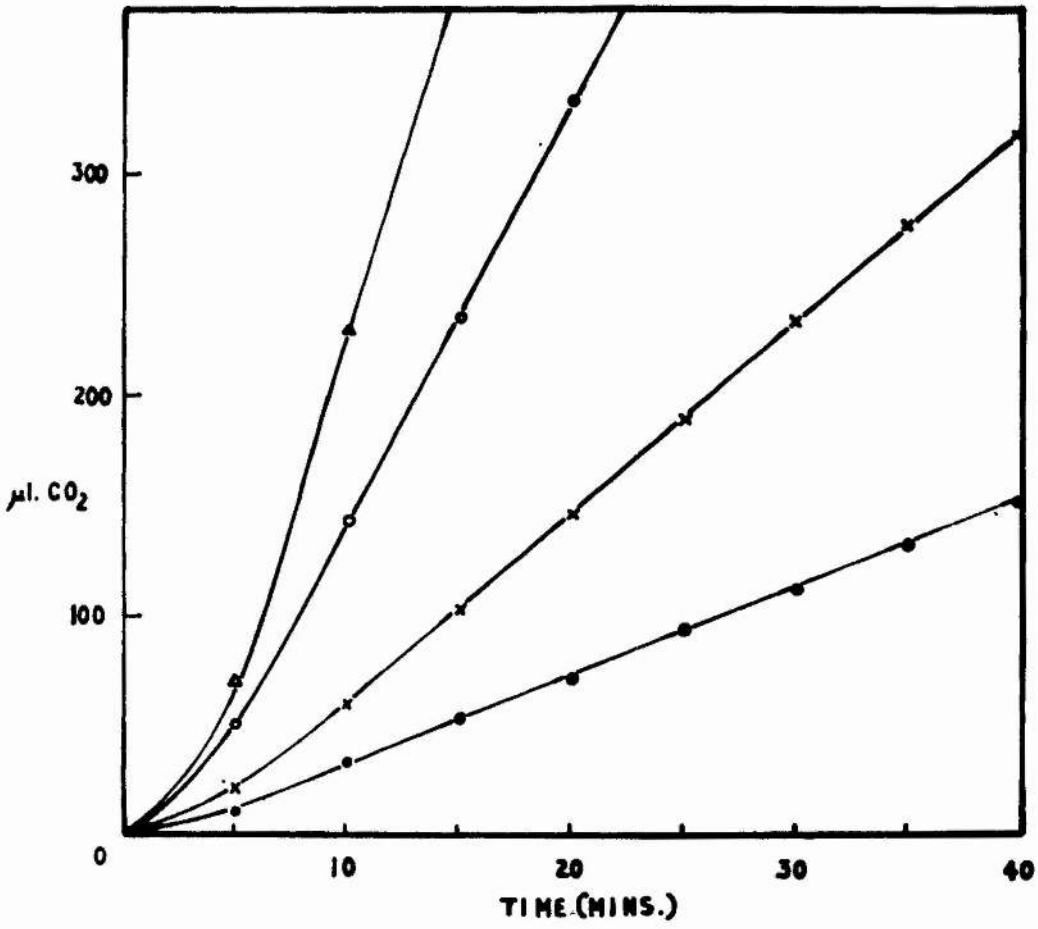
2.3. Experimental.

2.3.1. Concentrations of Yeast and Substrate.

Preliminary experiments were carried out to determine the correct amounts of yeast and substrate which should be present to saturate the enzyme surface with substrate. Most of the earlier investigators quote yeast amounts in g./100ml., but do not differentiate wet weight and dry weight.

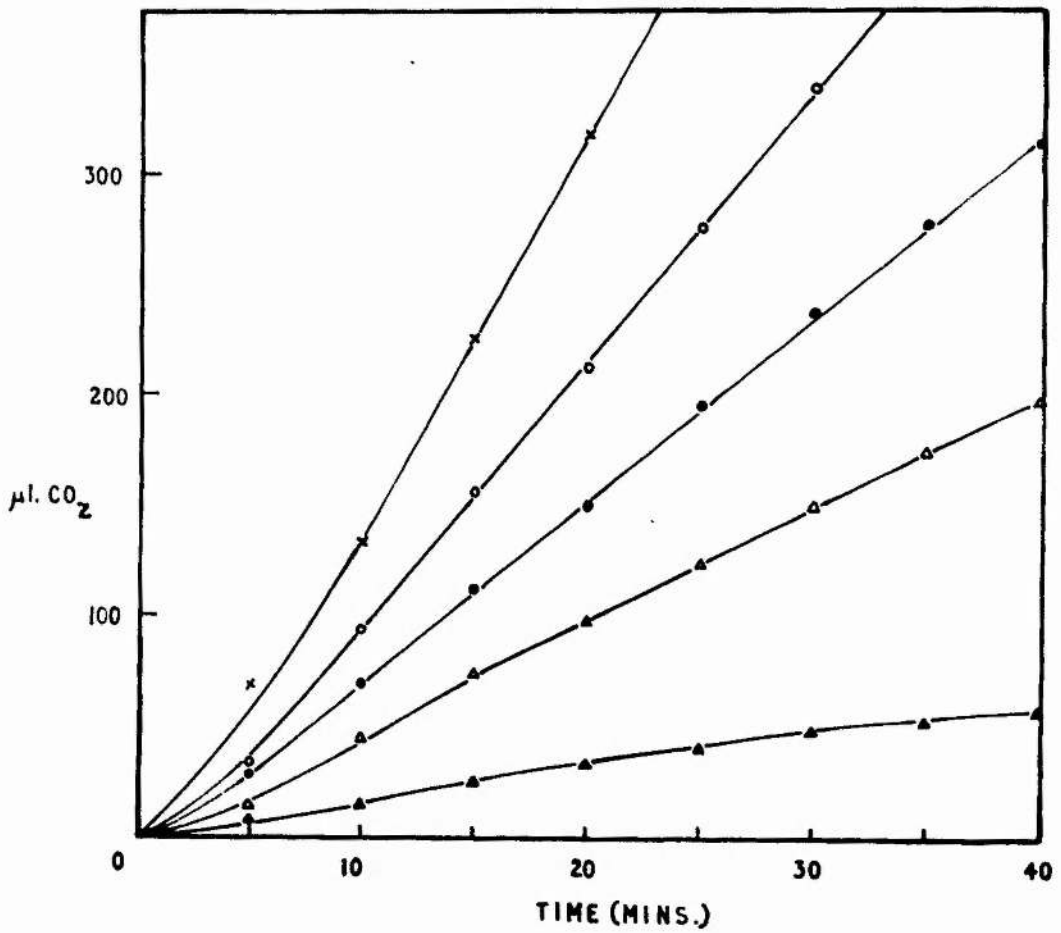
Hopkins and Roberts (1935) used a 0.1 per cent. suspension of pressed bakers' yeast, together with 0.005-0.84M hexoses when determining the Michaelis constants for glucose and fructose. They pointed out, however, that a 5.5 per cent. yeast suspension fermenting 0.004-0.02M glucose gives results which can be interpreted in terms of a unimolecular reaction. Rothstein and Cenza-Demis (1953) stated that 0.1M hexose is required to saturate completely the enzyme surface presented by 6.0-7.0mg. of yeast (wet wt.) but used concentrations down to 0.0025M when determining the K_S for glucose by the method of Lineweaver and Burk (1934) (Hurwitz and Rothstein, 1951). The lower concentrations of substrate are also used by Cramer and Woodward (1952) and Woodward and Hudson (1953) when determining the K_S for glucose, 2-deoxy glucose and glucosamine. 8.0mg. (wet wt.) of pressed bakers' yeast was used to ferment 0.01M

Fig.I. Effect of yeast concentration on fermentation rate.



D-Glucose, 0.025M. Yeast wet wt./3ml.: Δ–Δ, 40mg.; o–o, 25mg.; x–x, 10mg.; ●–●, 5mg.

Fig.II. Effect of D-glucosone on glucose fermentation.



Yeast, 25mg. wet wt./3ml; D-glucose, 0.025M; KCN, 2.6×10^{-4}M. D-glucosone: ×—× , none; o—o, 0.050M; ●—● , 0.100M; ▲—▲ , 0.150M; ▲—▲, 0.175M.

substrate. All of these investigators have been able to calculate the dissociation constant of the enzyme-substrate complex by assuming that the reaction conforms to the Michaelis-Menten equation. It is clear, therefore, that results can be obtained, for a wide range of yeast and substrate concentrations, which can be interpreted in terms of a monomolecular reaction in which an enzyme surface is saturated completely by the substrate present.

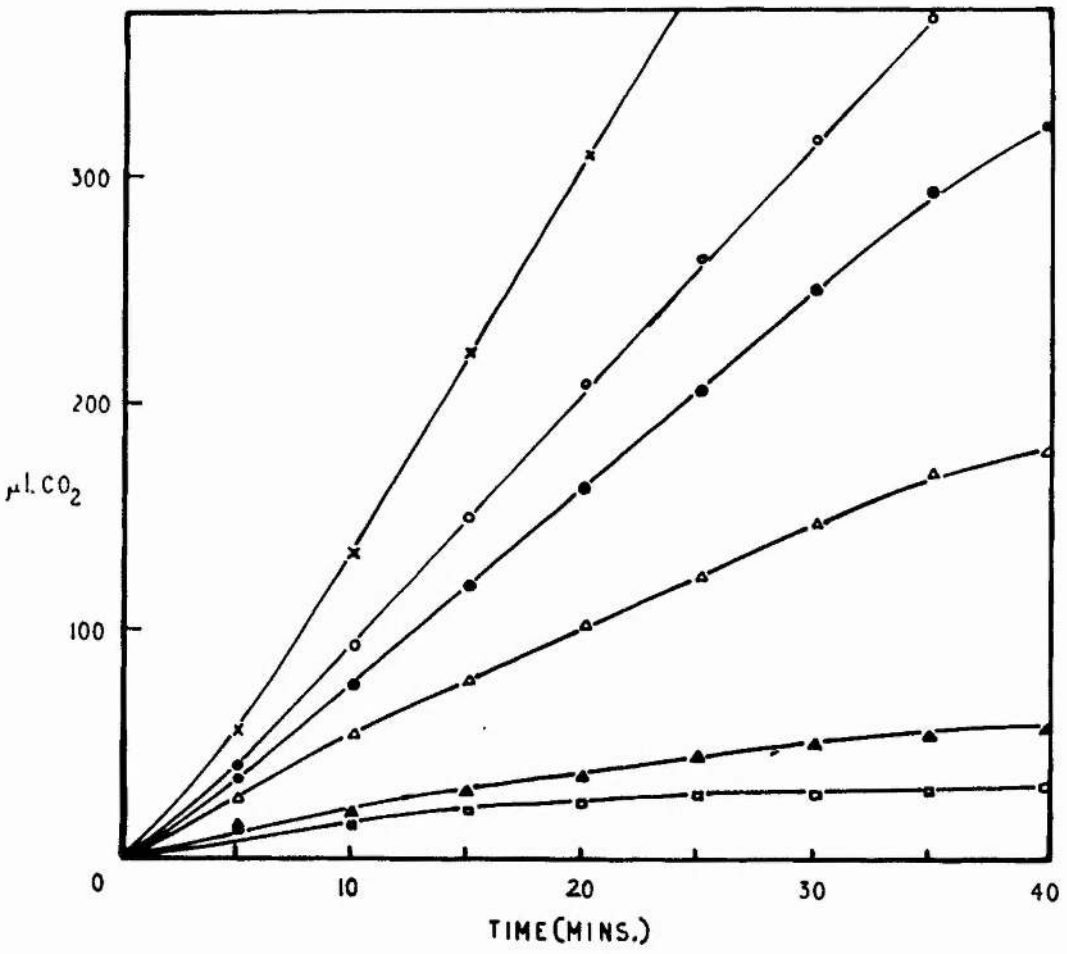
The effect of yeast concentration on fermentation rate is shown in Fig.I. 25mg. (wet wt.) pressed yeast was used with 0.025M glucose in the earlier experiments. Later, however, the amounts of yeast and substrate were modified to 10mg. (wet wt.) and 0.01M, to permit a wider range of inhibitor concentration.

2.3.2. Inhibition by Glucosone in the Presence of KCN.

The cyanide was added to each individual Warburg flask in the first experiments, but the inhibition of respiration was found to vary in individual flasks. The cyanide-treated suspensions were thereafter prepared as described in 2.2.

It was found that, using 0.025M glucose, the inhibition varied with the amount of added inhibitor (Fig.II.). That the inhibition was not a function of the

Fig.III. Glucose fermentation. Effect of constant inhibitor concentration on decreasing substrate concentrations.



Yeast, 25mg. wet wt./3ml.; D-glucosone, 0.10M; D-glucose; ×—×, 0.025M, no glucosone; □—□, 0.010M; ▲—▲, 0.015M; Δ—Δ, 0.020M; ●—●, 0.025M; ○—○, 0.030M.

absolute amount of inhibitor present was demonstrated in another experiment. (Table I.).

The ratio of inhibitor to substrate was kept constant at 4 to 1, and the rate of fermentation per hour is calculated from the carbon dioxide output for the period 0-20 minutes.

Table I.

Glucose (M)	Rate of Fermentation µl CO_2/hour		% Inhibition
	Substrate	Substrate + Inhibitor	
0.010	780	399	46
0.025	948	459	48
0.040	1084	522	48
0.050	1242	609	47

(Data from Appendix, Table 3).

The effect of decreasing the glucose concentration while keeping the glucosone concentration at 0.10M is shown in Fig.III.; the degree of inhibition is increased.

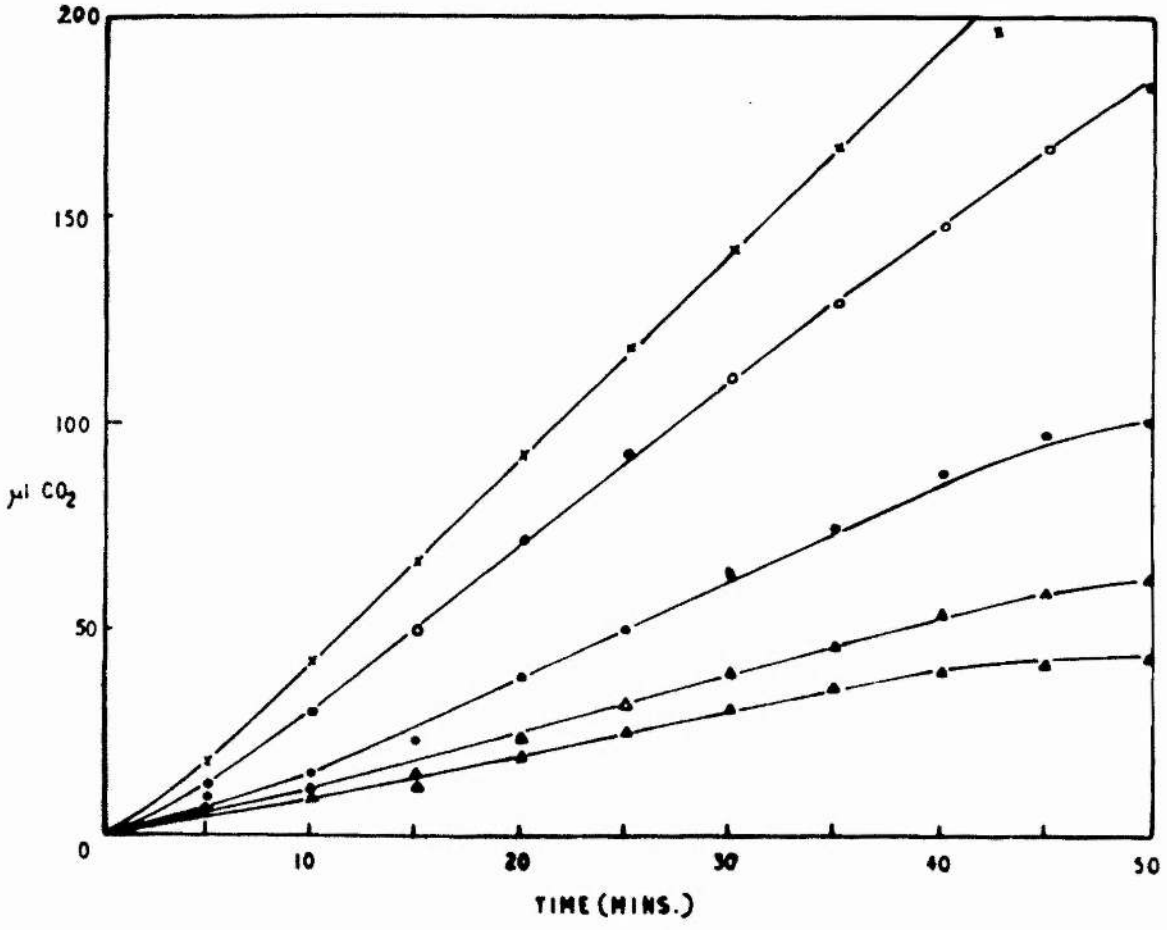
These results, when considered together, suggested that the inhibition produced was competitive in nature. It was not found possible, however, to confirm this competitive inhibition by kinetic analysis of the results, using the method of Lineweaver and Burk (1934).

As glucosone is known to be a highly reactive compound, it was considered possible that some chemical side reaction might influence the inhibition. The formation of a cyanohydrin by combination of an osone and aqueous cyanide was used by Ault, Baird, Carrington, Haworth, Herbert, Hirst, Percival, Smith, and Stacey (1933) and Reichstein, Grüssner, and Oppenhauer (1933) in the synthesis of ascorbic acids, but it seemed doubtful that this reaction could occur at the pH of fermenting yeast. The findings of Hynd (1927a) do suggest, however, that a combination of glucosone and potassium cyanide can occur at about pH 7, for it was reported that subcutaneous injection of glucosone into rats and mice afforded protection against the toxic effects of injected potassium cyanide, or inhaled gaseous hydrogen cyanide.

Should a combination of glucosone and cyanide take place in the system used, the effects would be very complex. Removal of cyanide could produce a simulated inhibitory effect by allowing respiration, and removal of glucosone a decreased inhibition of fermentation.

All further measurements of anaerobic fermentation were carried out in an atmosphere of nitrogen.

Fig.IV. Glucose fermentation. Effect of increasing inhibitor concentration at constant substrate concentration.



Yeast, 10mg. wet wt./3ml.; D-glucose, 0.01M; D-glucosone: ×—×, none; o—o, 0.010M; ●—●, 0.025M; Δ—Δ, 0.050M; ▲—▲, 0.075M.

2.3.3. Inhibition by Osones in the Presence of Nitrogen.

2.3.3.1. Using whole yeast cells.

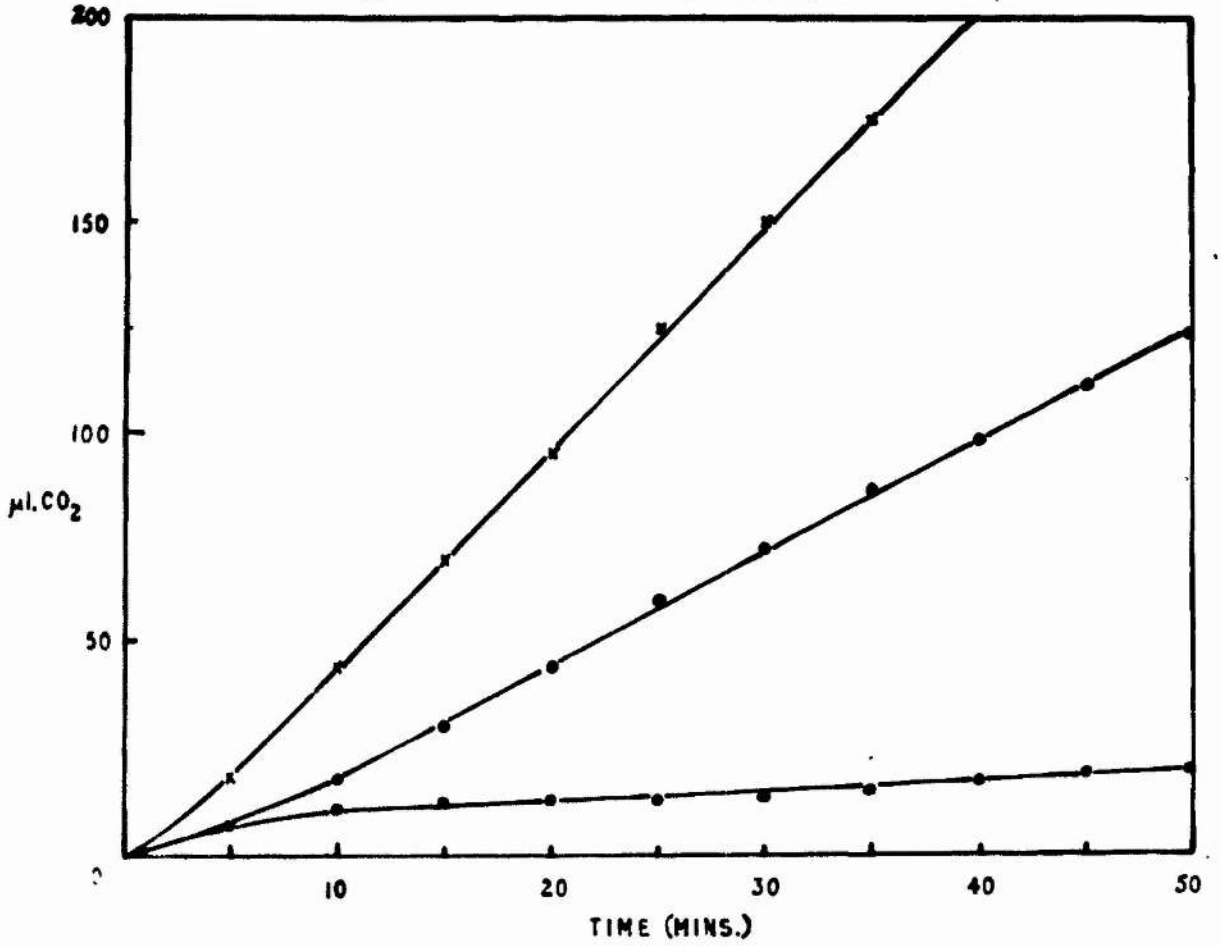
The inhibition produced by various concentrations of glucosone on the fermentation of glucose is shown in Fig.IV. The same general inhibitory effect is demonstrated as was shown when cyanide was used as the inhibitor of respiration. A more critical comparison of the results shows that the cyanide had a definite effect on the amount of inhibition produced by the osone, (Table II.). Here the fermentation rate is calculated from the 10-40 minute portion of the experiment, when the rate of carbon dioxide output is linear.

Table II.

Glucose (M)	Glucosone (M)	Fermentation rate		% Inhibition	
		KCN	N_2	KCN	N_2
0.01	-	284	308	-	-
0.01	0.010	204	238	27	23
0.01	0.025	110	146	61	52
0.01	0.050	52	90	82	71
0.01	0.075	20	64	93	79

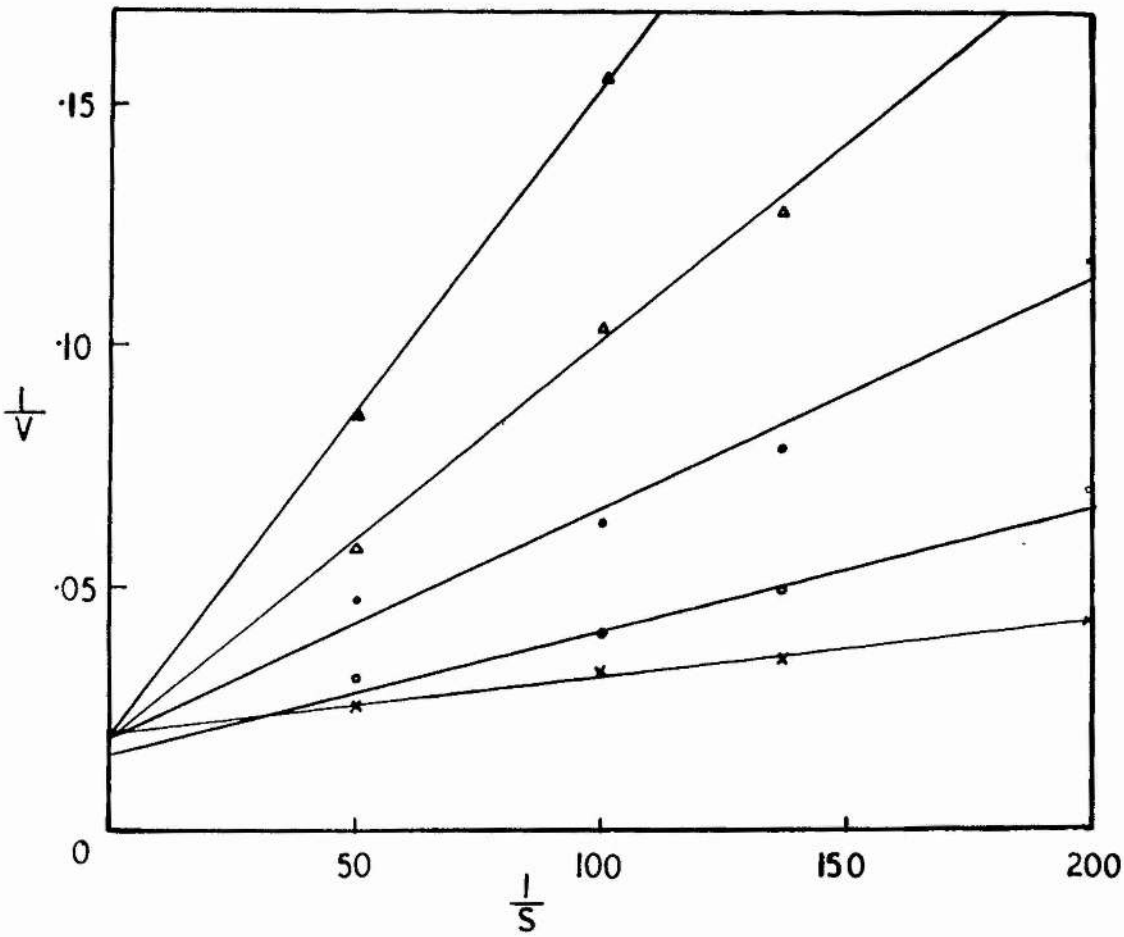
(Data from Appendix, Tables 5 and 6)

Fig.V. Effect of D- and L-glucosone on glucose fermentation.



Yeast, 10mg. wet wt./3ml.; x—x , 0.01M D-glucose; 0.01M D-glucose + 0.010M L-glucosone, and 0.01M D-glucose + 0.025M L-glucosone; o—o , 0.01M D-glucose + 0.010M D-glucosone; ●—● , 0.010M D-glucose + 0.025M D-glucosone.

Fig.VI. Competitive inhibition of glucose fermentation by D-glucosone.



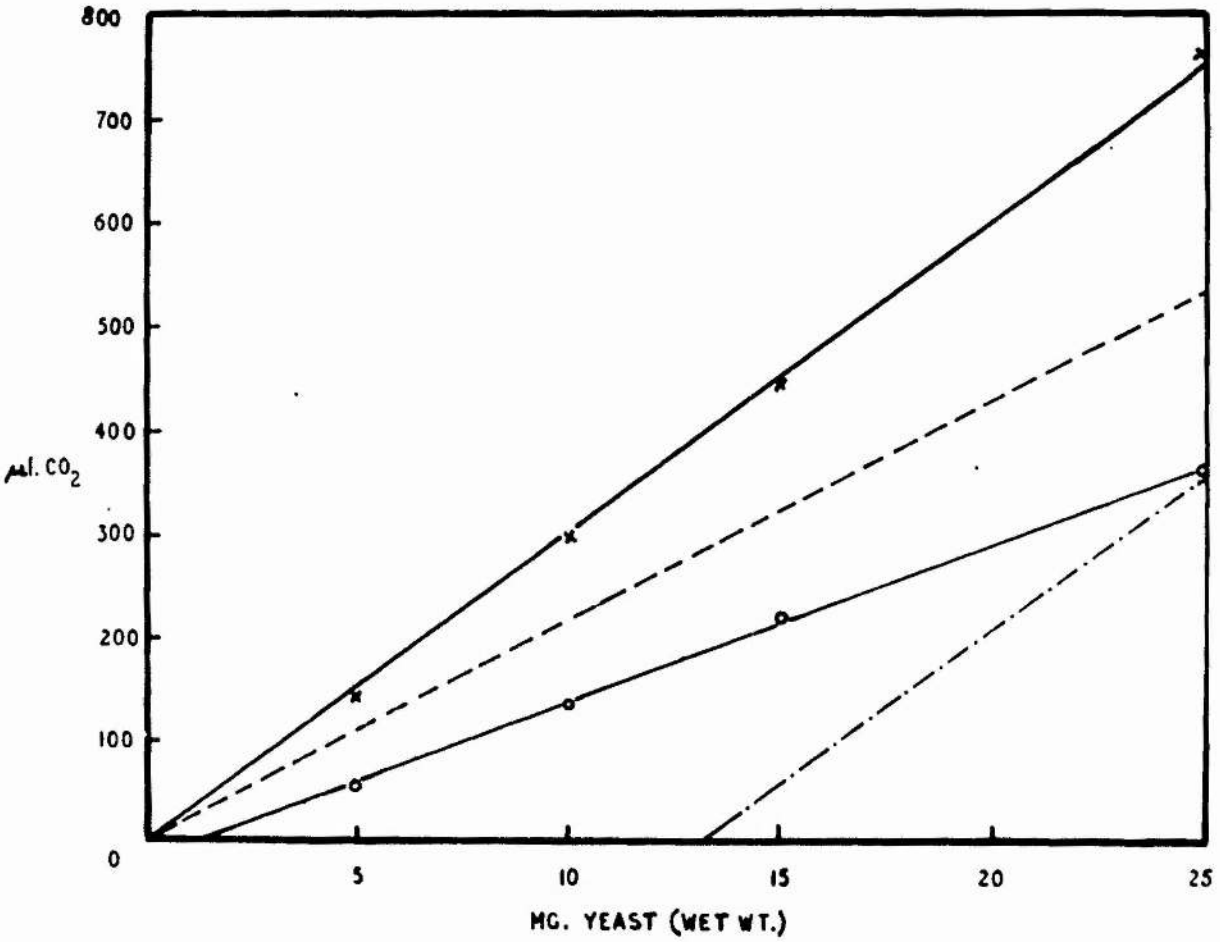
The curves shown are calculated by the method of least squares from the plotted experimental results. v, rate of CO_2 output per hour; S, molar substrate concentration. ×—× , no glucosone; ○—○ , 0.010M D-glucosone; ●—● , 0.025M D-glucosone; Δ—Δ , 0.050M D-glucosone; ▲—▲ , 0.075M D-glucosone.

The apparently greater inhibition shown in the experiments where cyanide was present is probably an artefact produced by combination of the cyanide with the osone. The consequent increase in respiration would then appear as a reduction in fermentation under the experimental conditions used.

The specificity of the inhibitory effect produced by glucosone is shown in Fig.V. L-glucosone, prepared and purified by a procedure identical with that used for D-glucosone showed no inhibitory effect even at a concentration at which D-glucosone produced complete inhibition.

A more comprehensive study of the inhibitory effect of glucosone was now made. Commercial dried yeast pellets were used in this part of the investigation, as this material provides readily available suspensions of yeast having a constant fermentative power. The collected results of these experiments are analysed in Fig.VI. The curves drawn are calculated by the method of least squares, the points plotted in the figure being based on experimental results. The curves cut the ordinate at the same point as the control and their slope increases with the inhibitor concentration. The inhibition produced by glucosone would seem, from these results, to be purely competitive in nature.

Fig.VII. Effect of yeast concentration on D-glucosone inhibition of glucose fermentation.



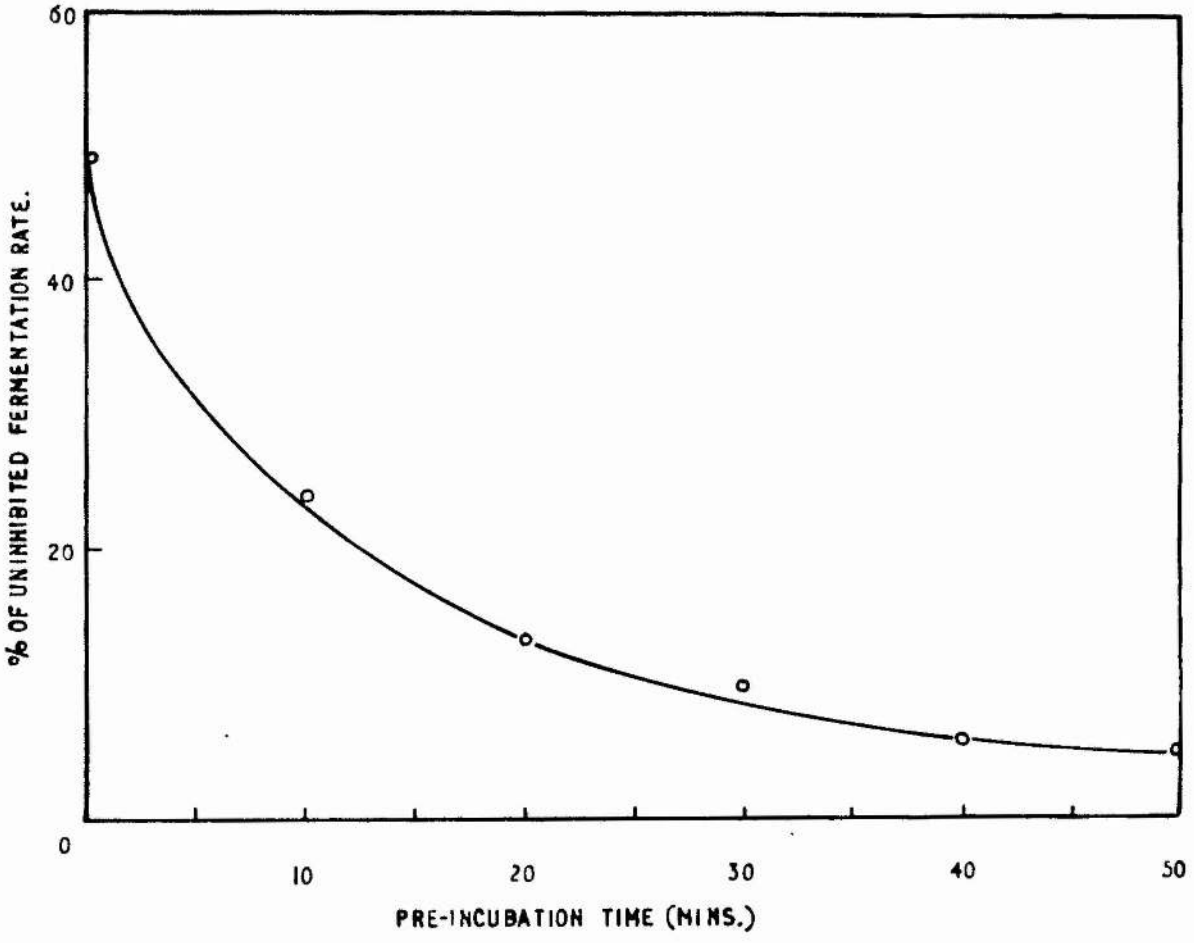
Yeast, mg. wet wt./3ml.; ×—× , 0.010M D-glucose; o—o , 0.010M D-glucose + 0.03M D-glucosone: – – – – , hypothetical curve for a reversible inhibitor of glucose fermentation; —·—·—· , hypothetical curve for an irreversible inhibitor of glucose fermentation.

This purely competitive effect could be demonstrated only in the 10-40 minute period. The non-linear induction period lasting up to 10 minutes is expected and observed with control experiments containing no inhibitor. The gradual increase of inhibition after 40 minutes cannot be due to any relative lowering of the glucose concentration since the rate of fermentation remains linear after this time when no inhibitor is present. It must be due, therefore, to the production, by glucosone, of an effect other than the competitive one described.

The true competitive inhibitor is by definition completely reversible in its attachment to the enzyme. In some cases, it is possible to demonstrate this reversibility by simple dialysis. The complex nature of the system used in the present experiments makes this method of investigating the nature of the enzyme-glucosone attachment impracticable, but Ackermann and Potter (1949) have described two simple tests which differentiate reversible and irreversible inhibitors.

In the first of these tests the rate of reaction is determined at different enzyme concentrations, in the presence or absence of inhibitor. When fermentation rate is plotted against enzyme amount, characteristic curves for substrate, reversible inhibitor and irreversible inhibitor are obtained. The application of the test to the present work is shown in Fig.VII.

Fig.VIII. Effect of yeast-glucosone pre-incubation on the amount of inhibition.



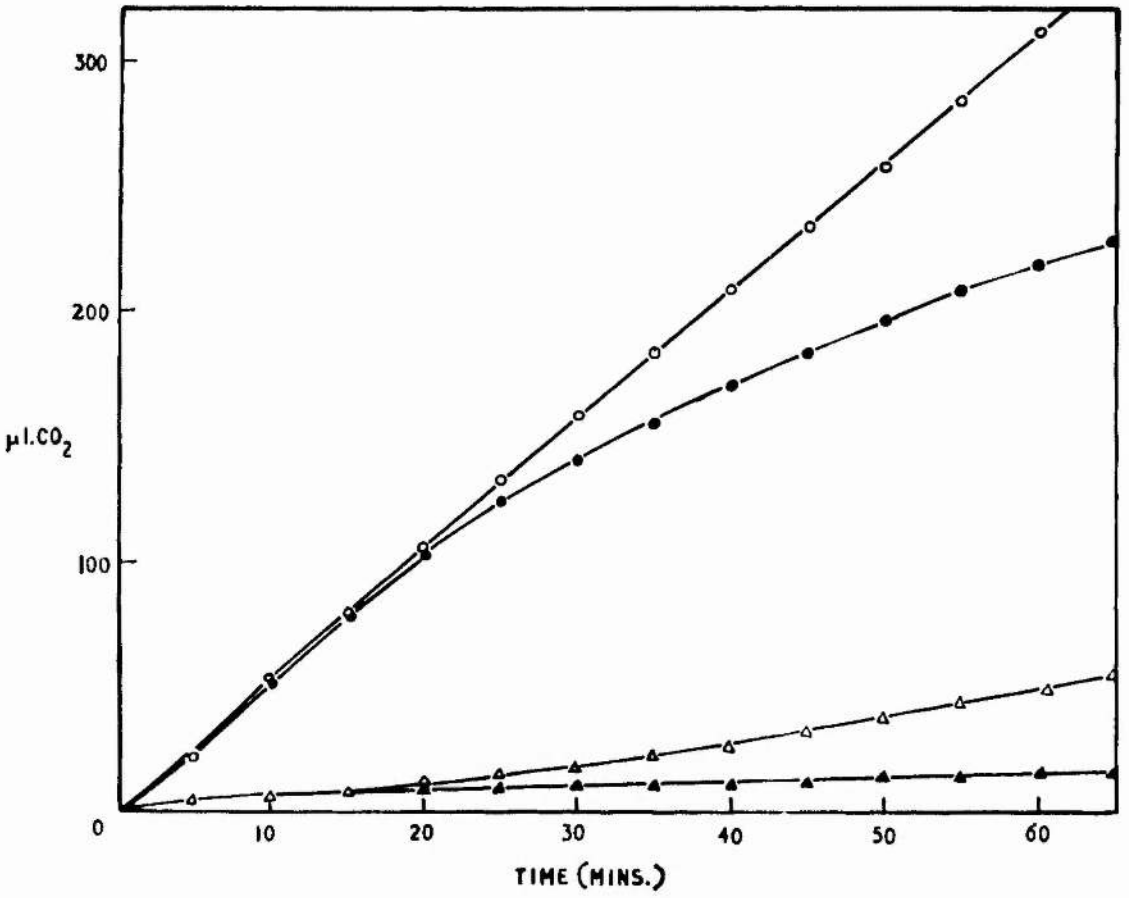
Yeast, 10mg. wet wt./3ml.; D-glucose, 0.01M; D-glucosone, 0.025M. Pre-incubation time is the length of time the yeast remains in contact with the inhibitor before the addition of substrate.

The rate of fermentation of 0.01M glucose by various concentrations of yeast was determined in the presence and absence of 0.03M glucosone. The curve for the osone-inhibited fermentation is shown to have properties intermediate between those of a reversible inhibitor and an irreversible inhibitor (hypothetical examples are illustrated). It would appear, therefore, that glucosone falls into the "pseudo-irreversible" category of Ackermann and Potter (1949), having an extremely small enzyme-inhibitor dissociation constant. This type of inhibitor is discussed more fully in section 2.5.5.

These authors describe a second test in which the the effect of time duration of enzyme-inhibitor contact on the degree of inhibition is determined. Warburg flasks with two side limbs were used in this experiment, glucosone being placed in one limb, glucose in the other, and yeast suspension in the main compartment. The osone was tipped at zero time and thereafter the glucose was added successively at 5 minute intervals. Control flasks were run in which the glucose and glucose plus glucosone were added to the yeast suspension at zero time.

The results shown in Fig.VIII. demonstrate that the amount of inhibition produced by this concentration of glucosone depends on the time that the yeast is pre-incubated with the inhibitor.

Fig.IX. Effect of D-glucosone on actively fermenting yeast and of D-glucose on glucosone-treated yeast.



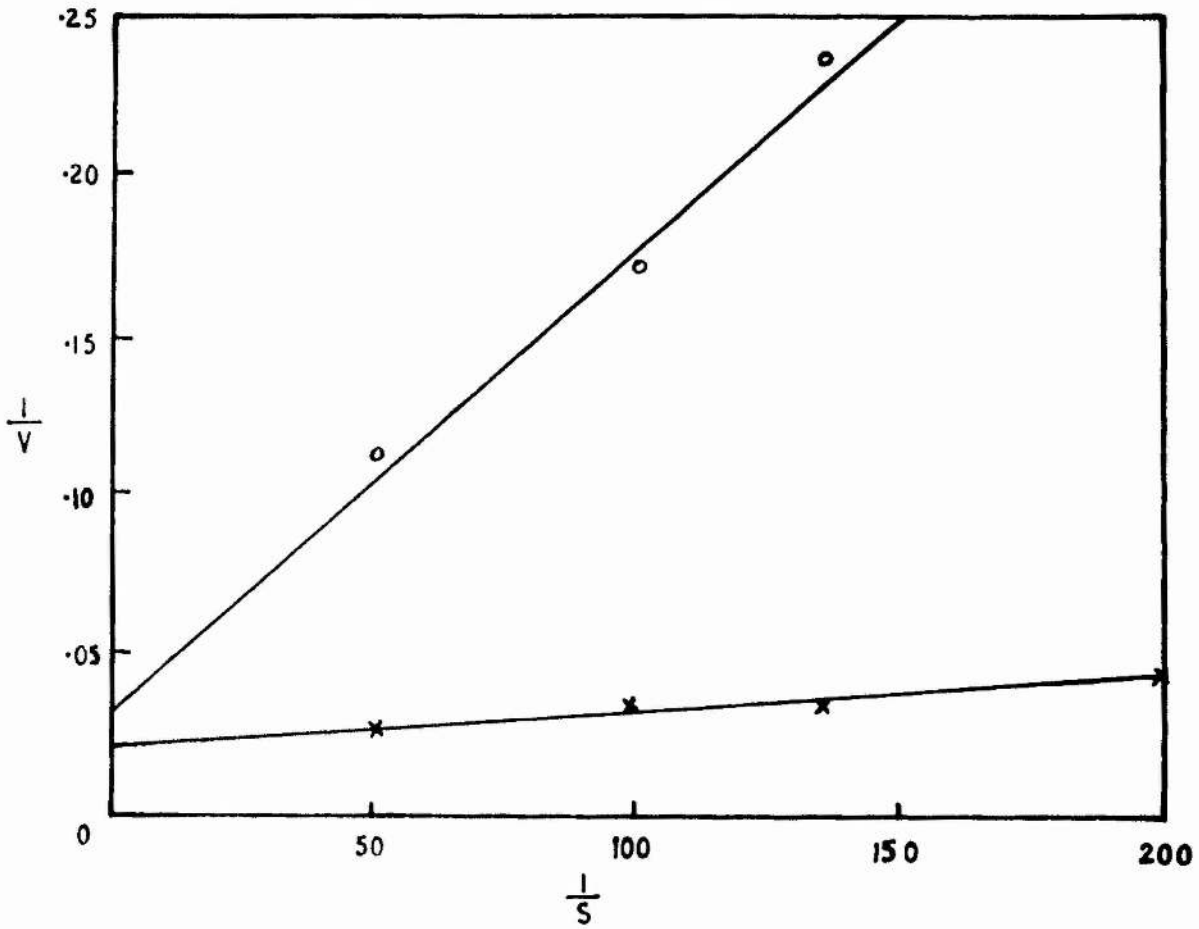
Yeast, 10mg. wet wt./3ml.; △—△ , 0.01M D-glucose added 15 minutes after 0.04M D-glucosone; ▲—▲ , 0.04M D-glucosone; ●—● , 0.04M D-glucosone added 15 minutes after 0.01M D-glucose; ○—○, 0.01M D-glucose.

The conclusion drawn from these tests, namely, that a very slowly reversible enzyme-inhibitor complex is formed, would explain the gradual increase on inhibition which has been observed (2.3.3.1.). The amount of glucosone combined with enzyme gradually increases until eventually complete saturation of the enzyme occurs. At this stage no further fermentation should occur.

Goldstein (1951) has described a test by which it is possible to measure the speed at which an enzyme-inhibitor-substrate complex attains equilibrium. This test also gives an indication of the rate at which the enzyme-inhibitor-complex dissociates. Warburg flasks with two side limbs were used in this experiment. In flask A, glucosone was added, and in flask B, glucose was added, to the yeast suspension at zero time. After noting the rate of fermentation for 15 minutes, glucose was added to flask A and glucosone to flask B. The rate of fermentation was then noted for a further 45 minutes.

Fig.IX. shows the results of one such experiment. The rates of fermentation tend to approach one another but within the limits of the experimental time never become equal. The inhibition caused by the addition of osone to the actively fermenting glucose is apparent much sooner than the small account of fermentation which occurs when glucose is added to the glucosone-yeast mixture. As the

Fig.X. Non-competitive inhibition of glucose fermentation by D-glucosone.



The curves shown are calculated by the method of least squares from the plotted experimental results. v, rate of CO_2 output per mg. wet wt. of yeast per hour; S, molar substrate concentration. ×—× , no glucosone; o—o, 0.025M D-glucosone.

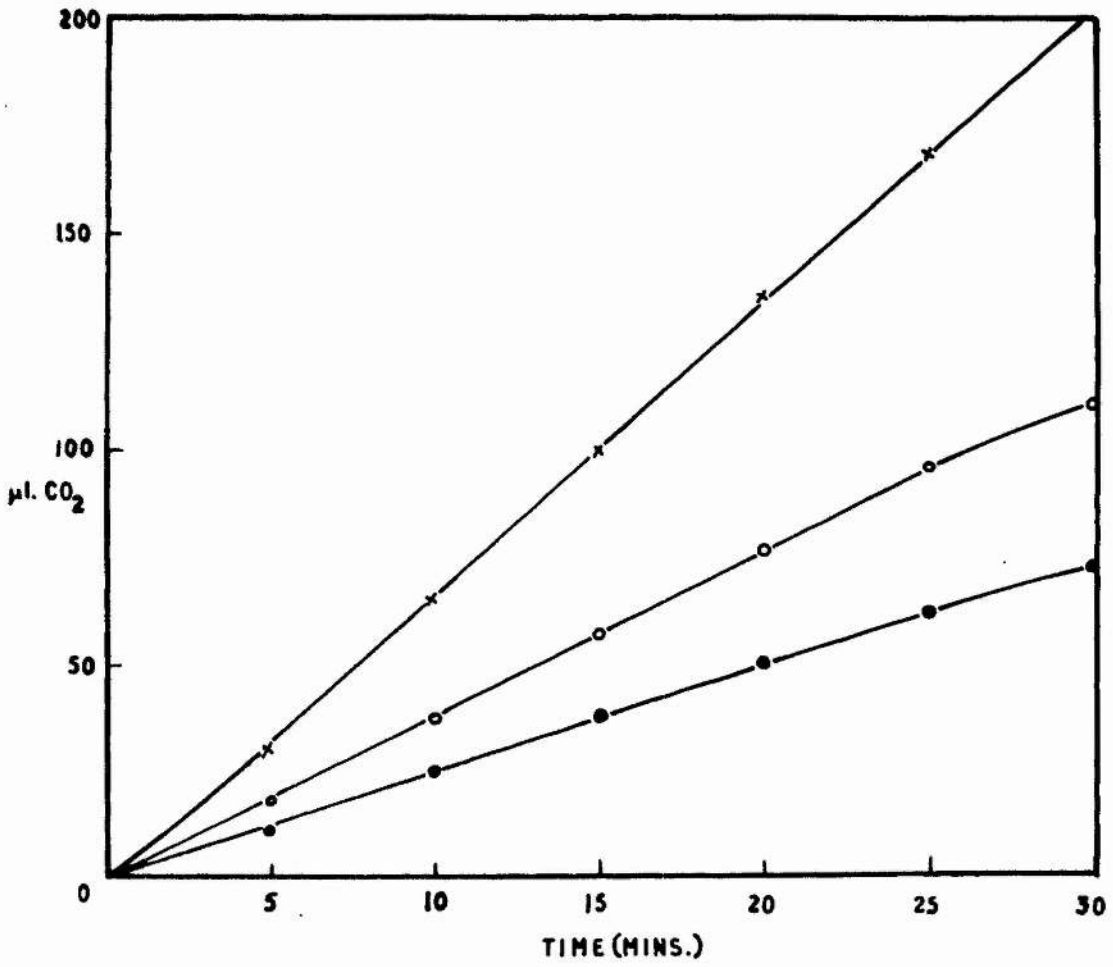
time taken for both flasks to reach the same equilibrated state is a measure of the speed of displacement of substrate by inhibitor, and vice versa, it is apparent that the removal of inhibitor by substrate is, in this instance, a much slower reaction than the removal of substrate by inhibitor.

As the effect of glucosone on yeast fermentation had been shown to vary with time, the type of inhibition exhibited by the osone after pre-incubation was investigated. Using Warburg flasks having two side limbs, glucosone was added to a yeast suspension at zero time, and ten minutes later, glucose was tipped in. The final concentration of glucosone was 0.025M, while that of glucose varied from 0.0075M to 0.020M. Controls without inhibitor were run at all concentrations of glucose.

The results of this experiment analysed in Fig.X., indicated that the inhibition was non-competitive in nature.

The experiments described so far gave results which suggested that the inhibitory effect displayed by glucosone on yeast fermentation changes, from a purely competitive inhibition to a non-competitive inhibition, with the formation finally, of an almost irreversible enzyme-inhibitor complex. This change would seem to be dependent on the time that the inhibitor is in contact with the yeast cell. Further aspects of these results are discussed in section 2.5.

Fig.XI. Effect of D-glucosone on glucose fermentation by dry ice yeast.



Yeast, 25mg. wet wt./3ml.; D-glucose, 0.01M; ×—× , no glucosone; o—o , 0.01M D-glucosone; ●—● , 0.02M D-glucosone.

2.3.3.2. Using dry ice yeast.

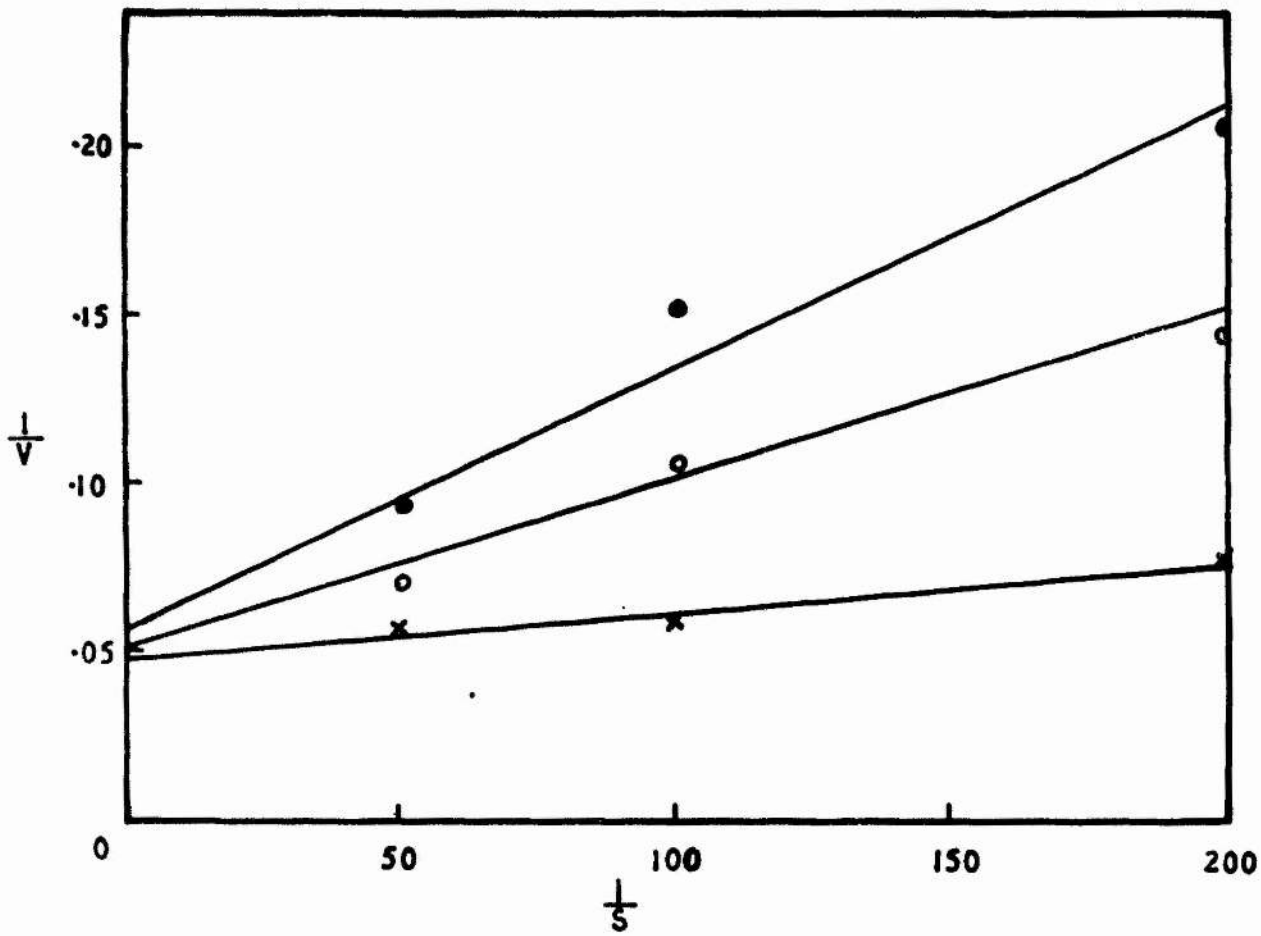
The effect of contact time on the nature of the inhibition could be explained in terms of different diffusion rates of substrate and inhibitor.

A study of the effects of glucosone in a cell-free fermenting system would show directly whether the cell membrane superimposed secondary effects on the general inhibitory pattern; however as the preparation of these extracts causes considerable disorganisation of the enzyme system cell-free extracts were not used in this part of the investigation.

A suitable fermenting system is provided by the dry ice yeast preparation (Part III. 3.3.), for it provides an enzyme source for fermentations in which the induction period is reduced to zero. Dixon and Atkins (1913) and Lynen (1939) have shown that permeability barriers can be overcome by exposing yeast cells to liquid air or liquid nitrogen. Krebs, Gurin, and Eggleston (1952) have also shown that the permeability of yeast cells to di- and tri-carboxylic acids is increased by less rigorous cold treatment. It is probable that any effects which may be produced on the present system by permeability barriers, will be removed by cold treatment.

The inhibitory effect of glucosone on the fermentation of glucose using dry ice yeast is shown in Fig.XI.

Fig.XII. Competitive inhibition of glucose fermentation by D-glucosone, using dry ice yeast.



The curves shown are calculated by the method of least squares from the plotted experimental results. v, rate of CO_2 output per mg. wet wt. of yeast per hour; S, molar substrate concentration. ×—×, no glucosone; o—o, 0.01M D-glucosone; ●—●, 0.02M D-glucosone.

The amount of yeast used in this experiment was greater than usual, in order to permit a rate of fermentation equal to that obtaining normally with 0.01M glucose. The osone exhibited a stronger inhibitory effect in this system, a ratio of osone to glucose of 5 to 1 resulting in complete inhibition of fermentation. This increased inhibitory effect could be explained by increased accessibility of the substrate and the inhibitor in this system. It was found that there was no initial fermentation lag period using cold-treated yeast, so that the rate of fermentation was measured over the period 0-20 minutes. The results were analysed by the method of Lineweaver and Burk (1934), in order to determine the type of inhibition. As the inhibition was competitive in nature (Fig.XII.), it was considered probable that the physical characteristics of the cell membrane do not exert any specific effect on the demonstrable inhibition.

It was considered possible, though improbable, that ATP might be a rate-limiting factor in the fermentation when osone was present in addition to glucose. A fermenting system which required ATP for full activity was obtained by keeping a dry ice yeast preparation at 0^{o} for 10 days. The effects of glucosone inhibition on glucose fermentation in the presence of limiting amount of ATP is shown in Table III.

Glucose and glucose plus glucosone were fermented in the absence, and in the presence, of different concentrations

of ATP. The inhibition is seen to be the same in each case.

Table III.

	ATP (M) -	0.001	0.006
Glucose, 0.01M.	405	453	519
Glucose, 0.01M + Glucosone, 0.01M.	171	207	207
% Inhibition	58	54	60

[μl CO_2]

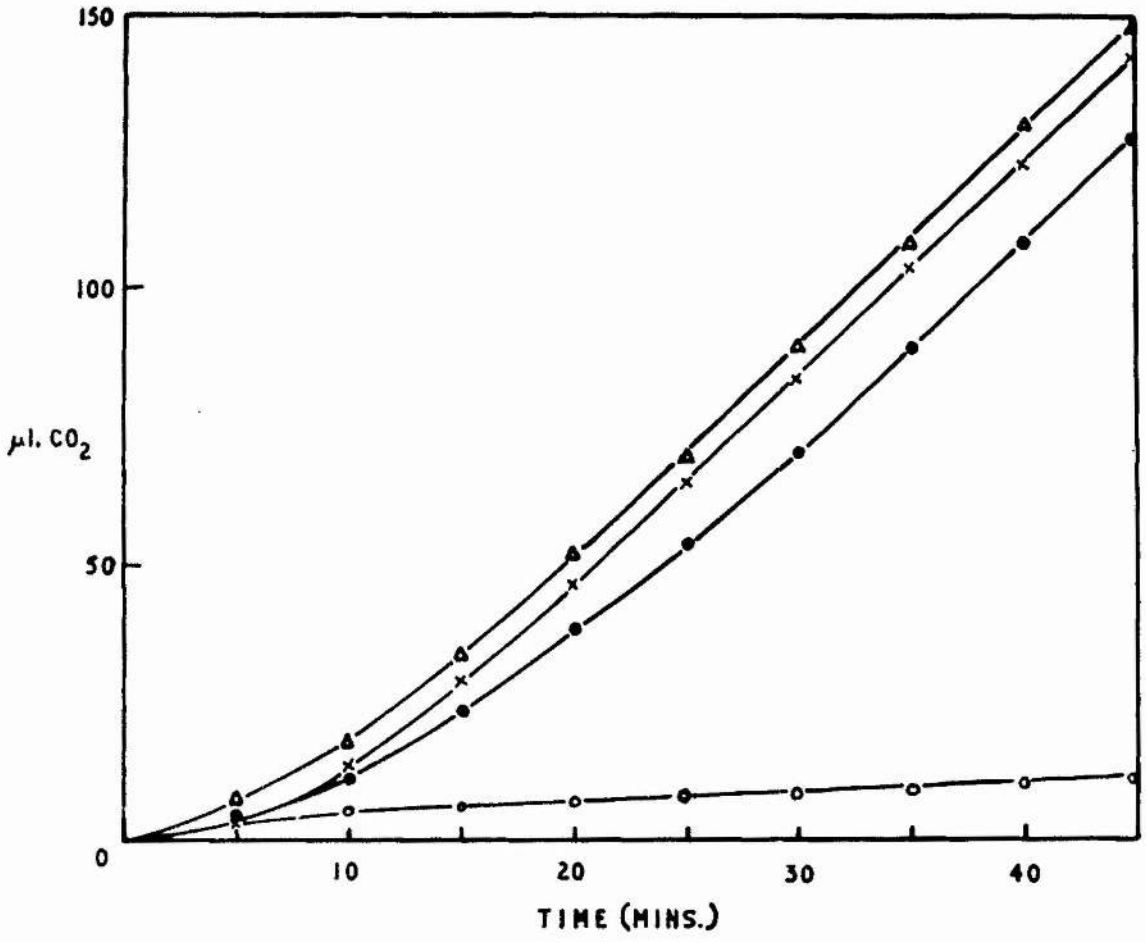
(Data from Appendix, Table 24)

These results demonstrate that the inhibition produced by glucosone is not affected by any permeability changes, nor is it dependent on the concentration of available ATP.

2.3.3.3. Using Cell-free Extracts.

The investigations on the inhibitory effects of glucosone on whole cell fermentation have shown that at some stage in the breakdown of glucose, glucosone, or some product of the further metabolism of glucosone, competes with a normal metabolite for any enzyme surface. In an attempt to locate this inhibition, the effects of glucosone

Fig.XIII. Effect of D-glucosone on the fermentation of fructose-1:6-diphosphate.



Cell-free yeast extract 1.0ml.; ×—× , 0.01M FDP and 0.01M FDP + 0.02M D-glucosone; Δ—Δ , 0.01M FDP + 0.04M D-glucosone; ●—● , 0.01M FDP + 0.08M D-glucosone; o—o , 0.08M D-glucosone.

on cell-free fermentation were studied.

A cell-free extract was prepared from dried bakers' yeast as described in Part III. 3.2. As shown in Fig.XIII., concentrations of glucosone up to 0.08M failed to show any significant effect on the fermentation of 0.01M fructose diphosphoric acid. This suggests that the inhibition occurs at some stage before the breakdown of diphosphoric acid.

The fermentation of 0.01M glucose was inhibited completely by 0.03M glucosone, using the cell-free extracts, as shown in Table IV. The increased inhibitory effect exhibited by the osone is probably due to the ease of combination of the enzyme and inhibitor in this disorganised system.

Table IV.

Glucose (M)	Glucosone (M)	% Inhibition
0.01	-	-
0.01	0.005	28
0.01	0.010	46
0.01	0.015	76
0.01	0.020	88
0.01	0.030	97

(Data from Appendix, Table 26)

Although the addition of ATP was necessary in order to obtain a satisfactory fermentation rate, further experiments showed that the ATP concentration was not limiting in the inhibited system (Table V.).

Table V.

	ATP (M)		
	0.003	0.006	0.012
Glucose, 0.01M.	204	184	206
Glucose, 0.01M + Glucosone, 0.01M	90	92	118
% Inhibition	46	50	43

[μl CO_2]

(Data from Appendix, Table 27)

It was not possible to determine the nature of the inhibition using the dry ice yeast preparation, or the cell-free extract, as accurate measurements of the changes of fermentation rate could not be made with varying substrate and inhibitor concentrations.

2.4. Discussion of Experimental Results.

2.4.1. The Site of Inhibition.

The results obtained in the fermentation experiments show that under certain defined conditions glucosone will compete with glucose for an enzyme in the glycolytic sequence. Gottschalk (1944), in confirmation of the results of Hopkins and Roberts (1935), concluded from the relationship between glucose concentration and the rate of metabolism, that the hexokinase-catalysed reaction was the slowest step in this process. On the basis of purely kinetic studies, it may be assumed, therefore, that the competition observed between substrate and inhibitor is produced in relation to hexokinase. The failure of glucose to inhibit the fermentation of fructose-1:6-diphosphate in cell-free extracts, supports this view.

Cramer and Woodward (1952) on similar grounds, have shown that 2-deoxy glucose inhibits glucose fermentation at the hexokinase stage, but it is possible that a phosphorylated derivative is the actual inhibiting agent. A direct proof of the site of the inhibition, and the nature of the actual inhibitor can be obtained only by the use of isolated purified enzymes.

The results obtained by Barron *et al* (1948) and Rothstein *et al* (1951) on the inhibition of yeast fermentation

by uranium, have been discussed earlier (1.2.). Hurwitz and Rothstein (1951) however, showed that the inhibition produced by uranium on fructose and glucose fermentation is competitive and non-competitive respectively. A specific fructose-uranium combination was also reported, so that the kinetic analysis of the uranium-inhibited fructose fermentation probably signified a competition between uranium and the cell for available fructose.

Rothstein et al (1951) reported that the amount of inhibition produced by uranium is dependent, to some extent, on the time of enzyme-inhibitor contact, for immediate inhibition was produced when uranium was added 10 minutes before, or at the same time as glucose, but when added 10 minutes after the glucose, the inhibition was not maximal for 60 minutes. The effects of greater enzyme-uranium pre-incubation times on the produced inhibitory pattern, were not reported however. As both Barron et al (1948), and Rothstein and Larrabee (1948), showed that uranium exerted its effect at the cell surface, it is possible that under the conditions used by Hurwitz and Rothstein, an amount of the cell surface was rendered impermeable towards glucose sufficient to produce non-competitive effects. In an earlier investigation, Slater and Sand (1910) calculated that diffusion of free glucose into the yeast cell was rapid enough to account for normal metabolic rates. The "active transport"

of glucose into the yeast cell as proposed by Rothstein *et al*, is therefore unnecessary, and the postulated location of a primary phosphorylating enzyme on the surface of the cell is not proven by the results presented.

Evidence has been presented by Cramer and Woodward (1952) which is considered to support the suggested location of a phosphorylating enzyme at the yeast cell surface. They showed that 2-deoxy glucose inhibited glucose fermentation by whole yeast cells but did not inhibit glucose fermentation by cell-free extracts during the preliminary 40-50 minutes of the experiment. The inhibition of whole cell fermentation was therefore considered to be due to the inhibition of a surface enzyme responsible for the passage of glucose into the cell. From its recognised rate-limiting action in glucose fermentation, it would appear that hexokinase is the enzyme concerned in this inhibition. It is therefore difficult to understand how cell-free fermentation is not inhibited by 2-deoxy glucose. As Cramer and Woodward also reported that ATP became rate-limiting after a 40-50 minutes fermentation in the presence of 2-deoxy glucose, using the cell-free system, it is possible that the concentrations of inhibitor used were insufficient to display any immediate significant effect. These results would seem to provide no further proof of the location of an essential glucose phosphorylating enzyme at the cell surface.

The present experiments show that glucosone, or a metabolic derivative of glucosone, competes with glucose for some enzyme in such a way that the ATP concentration is not a function of this inhibition. As it has also been shown that the inhibitory effect occurs in cell-free extracts, at non-limiting ATP concentrations, the effect is not due to the location of an enzyme or enzymes at the cell surface.

There is no evidence to show that 2-deoxy glucose and glucosone exert their inhibitory effect on yeast fermentation at the same point, although both are known to be phosphorylated by hexokinase (Cramer and Woodward, 1952; Johnstone and Mitchell, 1953). It is possible, therefore, that two different inhibitory effects are being demonstrated which give similar overall results in whole yeast cell fermentation.

Thus the site of the inhibition has not been definitely ascertained, although it is most probable that either hexokinase is inhibited directly by the osone, or in an indirect manner by a derivative of glucosone. The phosphorylation studies reported later tend to confirm this supposition.

2.4.2. <u>The Specificity of the Inhibition.</u>

The inhibitory effect is specific for D-glucosone as evidenced by the lack of inhibitory effect shown by the L-isomer. As the L-glucosone was prepared and purified

by a method identical with that used for D-glucosone, the inhibition produced by the latter is not due to any impurity present. Chromatographic analysis (Part III. 5.) was also used to check the homogeneity of the osone samples. Hynd (1927a) reached similar conclusions regarding the effect of D-glucosone, by showing that lactosone and maltosone had no effect on animals, whereas the products of acid hydrolysis of these osones gave results identical with the toxic effects shown by D-glucosone itself. The more comprehensive investigating of Bayne (1952), in confirming the specificity of the D-glucosone effect is considered in section 1.9.2.

Woodward and Hudson (1953) have shown that glucosamine inhibits glucose fermentation, although to a smaller extent than does 2-deoxy glucose. More recently Woodward, Cramer, and Hudson (1953) have reported that 2-chloro-2-deoxy glucose and iso-glucosamine both show some inhibitory effect towards glucose fermentation. The negative effect of a number of other glucose and fructose analogues on fermentation, was also reported.

It is possible to suggest, therefore, that those substances which inhibit glucose fermentation by yeast, do so by reason of a structural change on C2 of the natural substrate. It is also possible that these inhibitors combine with the same enzyme to produce their inhibitory effect.

2.5. Kinetics.

2.5.1. Introduction.

A knowledge of the kinetics of enzyme reactions makes possible a classification of the known types of enzyme inhibition, and also some understanding of the mechanism of these reactions. For these reasons, enzyme reactions have been analysed kinetically almost as long as have purely chemical reactions.

One of the first accepted views on the velocity of enzyme reactions was based on the work O'Sullivan and Thompson (1890), who constructed time curves representing the velocity of the invertase reaction, and showed that the law of mass action held in this instance. This conclusion was confirmed by O'Sullivan (1892), who investigated the rate of sucrose inversion by whole yeast cells. These findings were based purely on the similarity between theoretically derived curves and thoseobtained experimentally.

When these results were subjected to a more rigorous analysis by Brown (1902), who calculated the constant k of the mass action equation for unimolecular reactions:

$$k = \frac{1}{t} \log. \frac{1}{1-x,} \qquad (1)$$

where x is the fraction of sugar inverted in time t, deviations from the mass action law were noticed. In an attempt to

explain these deviations, Brown considered the empirical equation:

$$2k = \frac{1}{t} \log. \frac{1+x}{1-x} \qquad (2)$$

put forward Henri (1901) which described correctly the reaction velocity of invertase; the findings of Wurtz (1880) who showed that papain appeared to form an insoluble compound with fibrin previous to its hydrolysis; and those of Fischer (1894, 1895) who demonstrated specific stereochemical requirements for the attachment of substrate and enzyme.

Brown (1902) suggested that an enzyme-substrate complex was formed which required a definite length of time to break down into products; therefore an increase in the substrate concentration beyond a certain limit, which was dependent on the enzyme capacity, could produce no further increase in reaction rate. The same view was put forward by Brown and Glendinning (1902), who suggested that this type of reaction was first order with respect to the enzyme-substrate complex rather than with respect to the substrate.

This theory was finally expressed by Michaelis and Menten (1913) as a mathematical relationship valid for a large range of substrate concentrations. The rate of the observed reaction was assumed to be directly proportional

to the concentration of the enzyme-substrate-complex [ES] at all values of the concentration of the substrate [S]. The numerical value of the dissociation constant, which in the simplest case at equilibrium may be expressed as:

$$K_S = \frac{[E]\ [S]}{[ES]} \qquad (3)$$

is numerically equal to the substrate concentration at half maximum velocity.

Michaelis and Menten (1913) assumed that only a small number of enzyme centres acted in normal enzyme catalysed reactions, and that they acted according to the laws governing first order reactions. They applied the law of mass action, assuming that the concentration of one component of the reaction, [E] + [ES], remained constant, and the other [S], varied. The observed initial velocity of reaction is then given by the equation:

$$v = V_{max.} \left(\frac{[S]}{K_S + [S]}\right) \qquad (4)$$

where V_{max} is a numerical constant representing the maximum velocity obtained when the enzyme E exists completely in the form ES, being thus proportional to the enzyme concentration. K_S is a constant characteristic of the enzyme.

As equation (4) is based on the rather fallacious assumption that the combination of enzyme and substrate is always in equilibrium, ie. that the velocities of formation and dissociation of these compounds are infinite in comparison with the decomposition velocity, Briggs and Haldane (1925) considered the situation when the velocities of the reactions:- $E + S \rightarrow ES$, $ES \rightarrow E + S$, and $ES \rightarrow E +$ products, were comparable. If the velocity constants are, k_1, k_2, and k_3, they showed that a more accurate representation of the reaction is given by the equation:

$$\frac{k_2 + k_3}{k_1} = \frac{[E] . [S]}{[ES]} \qquad \text{cf(3).} \qquad (5)$$

Thus, if $k_2 + k_3/k_1$ is equal to K_s the result is the same as that given by Michaelis and Menten. Haldane (1930) pointed out that the Michaelis constant need not, therefore, be an enzyme-substrate dissociation constant, although it has the dimensions of such a constant, expressed as a molar or percentage concentration, and may be for most purposes used as such.

A further step towards a more definitive method, of studying the kinetics of enzyme reactions was made by Hanes (1932). He measured by an "initial slope" method the velocity of starch hydrolysis in the presence of amylase, and observed that the relationship between initial

velocity and enzyme concentration was linear over a wide range of enzyme concentration, in agreement with Michaelis' theory. Hames (1932) was able to calculate by the method of least squares the V_{max} and the K_s of the reaction, from the initial velocity of the reaction at varying substrate concentrations.

Later, it was pointed out by Lineweaver and Burk (1934) that in many of the cases investigated the mechanism and equations of the simplest case, as given by Michaelis and Menten (1913), have been assumed to hold without regard to other possibilities. The dissociation constant K_s, true or apparent, was evaluated by plotting activity (initial velocity), against $[S]$ or log. $[S]$, and taking the value of $[S]$ at half maximal activity, without regard for the mechanism concerned in the reaction. These authors proposed a means whereby it would be possible to ascertain which of several mechanisms might, or might not, be involved. Six variations of the original equation:

$$[E] + [S] = [ES] \qquad (6)$$

were described, in which substrate activation, substrate inhibition, general competitive and non-competitive inhibition, and reactions of various orders were considered. The velocity in all of these cases was considered to be a direct function of the concentration of an active intermediate, or active intermediates.

A graphical method of testing velocity equations and evaluating constants involved in the postulated mechanisms was evolved. It consists in expressing the given equation in a form that is linear, which permits straight line extrapolations.

Normal first order reaction constants can be evaluated, for example, by taking the reciprocal of both sides of equation (4) to give:

$$\frac{1}{v} = \frac{K_s}{V_{max.}} \frac{1}{[S]} + \frac{1}{V_{max.}} \qquad (7)$$

When $1/v$ is plotted against $1/[S]$ in this case, the ordinate intercept is $1/V_{max.}$, and the slope of the straight line is $K_s/V_{max.}$ so that K_s can be evaluated. Hanes (1932) had previously based his method of evaluating K_s and $V_{max.}$ on the equation:

$$\frac{[S]}{v} = \frac{[S]}{V_{max.}} + \frac{K_s}{V_{max.}} \qquad (8)$$

which is, in fact, equation (7) multiplied through by S. In this case when $[S]/v$ is plotted against $[S]$ the ordinate intercept is $K_s/V_{max.}$ and the constant slope $1/V_{max.}$ Haldane (1930) also considered some of the more complicated enzyme reactions, and derived equations for them. The Lineweaver-Burk derivations are better, however, since the

final equation is in a linear form which allows statistical methods to be applied very readily.

2.5.2. Enzyme Inhibition.

Enzymes are proteins, and as such, contain free amino, carboxyl and sulphydryl groups. These groups may be blocked by various substances with a concomitant non-specific inhibition of enzyme activity which may be reversible or irreversible. Irreversible non-specific inhibition is usually brought about by agents which cause denaturation or destruction of the enzyme, so that very little information can be gained by using such substances. The study of reversible non-specific inhibitors, on the other hand, has produced much information regarding the nature of the groups essential for enzyme activity. The type of inhibition produced by such reversible inhibitors is termed 'non-competitive'. Enzymes are also considered to have other essential groups or 'active centres', which confer specificity on the enzymes. As substrates are assumed to combine with these active centres, it follows that any substance reacting with these essential groups will inhibit the enzyme. Specific inhibitors such as these, which are reversible, are termed 'competitive' inhibitors.

The treatment of competitive and non-competitive inhibition by kinetic analysis will be considered here in relation to the present work.

2.5.3. Competitive Inhibition.

The simplest case of competitive inhibition may be written, according to Lineweaver and Burk (1934), as:

$$\frac{1}{v} = \frac{1}{V_{max.}}\left[\left(K_s + \frac{K_s[I]}{K_i}\right)\frac{1}{[S]}\right] + \frac{1}{V_{max.}} \qquad (9)$$

where $[I]$ is the concentration of the inhibitor, and K_i the enzyme-inhibitor dissociation constant. The term $K_s[I]/K_i$ represents an increase of slope in the $1/v$ against $1/[S]$ plot when $V_{max.}$ remains constant.

Ebersole, Guttentag, and Wilson (1944) have pointed out that equation (9) can be written:

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\cdot\frac{[I]}{K_s + [S]} \qquad (10)$$

where v is the velocity of the reaction in the absence of the inhibitor, and v_i is the velocity of the reaction in the presence of the inhibitor. A transformation of this equation was introduced by Hunter and Downs (1945) by substituting the fractional activity v_i/v by a, so that:

$$[I]\cdot\frac{a}{1-a} = K_i + \frac{K_i}{K_s}[S] \qquad (11)$$

When $[I] . a/1-a$ is plotted against $[S]$, the resulting straight line curve intercepts the ordinate at a value equal to K_i, its slope being equal to K_i/K_s. $[I] . a/1-a$ is numerically equal to the concentration of inhibitor producing an inhibition of 50 per cent., and varies with the concentration of substrate.

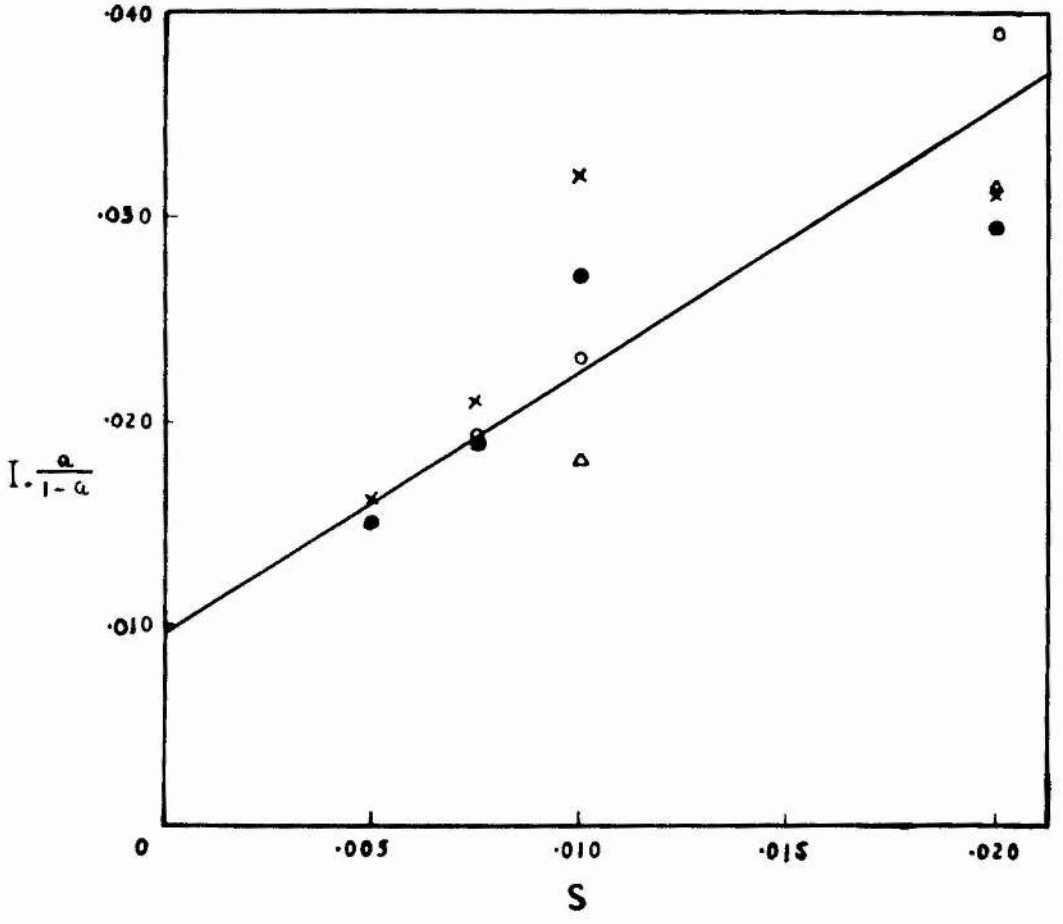
Equations (9) and (11) therefore, provide means whereby competitive inhibition can be recognised graphically.

In Fig.VI. $1/v$ is plotted against $1/S$ using data from tables 7-11 of the Appendix. v, the velocity of reaction is the rate of carbon dioxide output per hour per 10mg. yeast (wet wt.), calculated from the period 10-40 minutes of the fermentation reaction, expressed in cub. mm. carbon dioxide. $[S]$ is the molar concentration of substrate present and $[I]$ the molar concentration of glucosone varied as described in Fig.VI.

The K_s for glucose, for this system, is 6.0×10^{-3}M and K_i the enzyme-inhibitor dissociation constant is 7.5×10^{-3}M. Glucosone displays true competitive inhibition under these conditions, for $V_{max.}$ remains constant, only the slope of the line increasing. This increase in slope of the inhibitor curves is very similar to that expected from theoretical considerations, ie. by an amount equivalent to

$$1 + \frac{[I]}{K_i} .$$

Fig.XIV. Competitive inhibition of glucose fermentation by D-glucosone.



The curve shown is calculated by the method of least squares from the plotted experimental results. a, degree of inhibition of fermentation rate; I, molar concentration of inhibitor; S, molar substrate concentration; x-x, .010M D-glucosone; •-•, .025M D-glucosone; o-o, .050M D-glucosone; Δ-Δ, .075M D-glucosone.

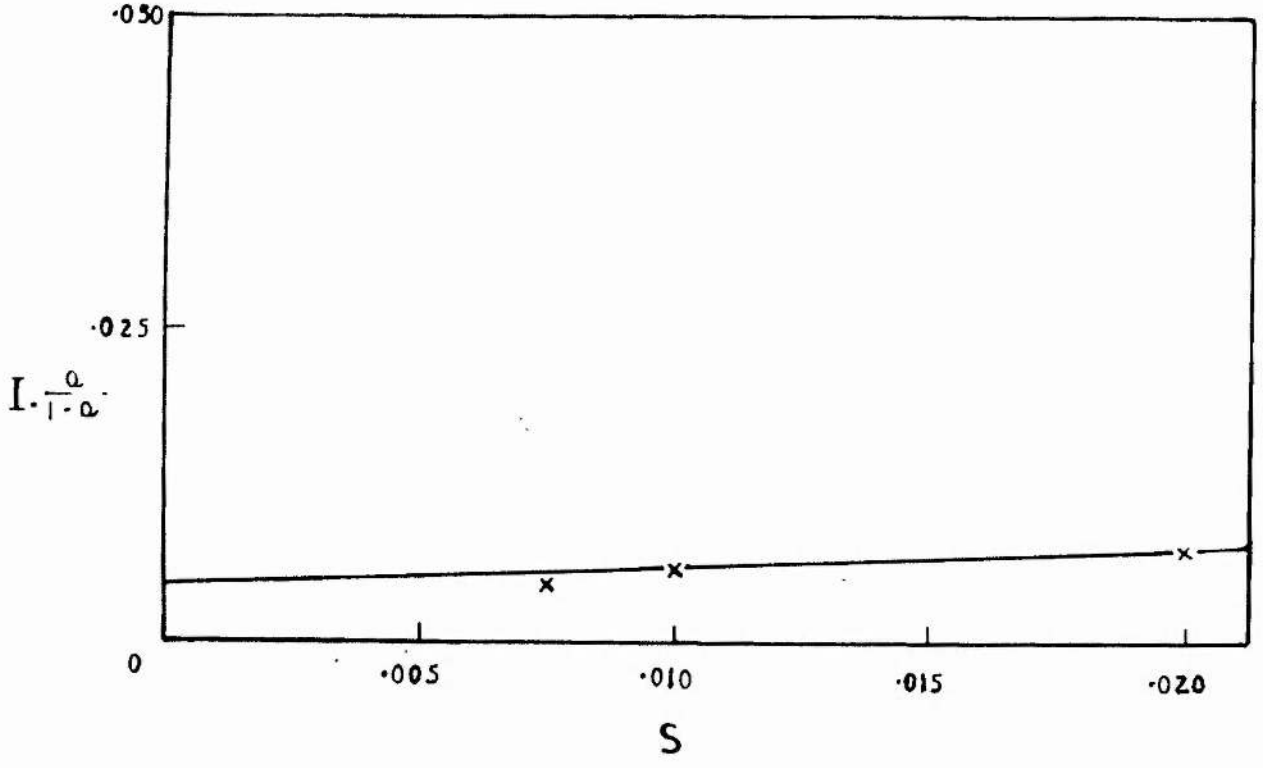
Similarly, a plot of $[I] \cdot a/1-a$ against $[S]$ shown in Fig.XIV., using data from table 7-11 and 28, of the Appendix, shows that the inhibition exhibited by D-glucosone on the fermentation of D-glucose is competitive in nature. The intercept on the ordinate, being equal to K_i, is 9.6×10^{-3}M which compares well with that obtained by the previous method. The slope of the line is K_i/K_s, so that the K_s for glucose, for this system, can be calculated as 7.5×10^{-3}M.

2.5.4. Non-Competitive Inhibition.

The degree of enzyme inhibition in the case of non-competitive inhibitors is dependent, by definition, solely on the amount of enzyme combining with the inhibitor, there being no competition between the inhibitor and substrate.

Non-competitive inhibition is described by an equation similar to that for competitive inhibition, according to Lineweaver and Burk (1934). When $1/v$ is plotted against $1/S$ a curve is obtained which has a slope increased above that of a control by $1 + [I]/K_i$, and also an intercept on the ordinate increased by this same amount. The value of $V_{max.}$ therefore, varies inversely as the inhibitor concentration.

Fig.XV. Non-competitive inhibition of glucose fermentation by D-glucosone.



The curve shown is calculated by the method of least squares from the plotted experimental results. a, the degree of inhibition of fermentation; I, molar concentration of inhibitor; S, molar substrate concentration. ×—× , 0.025M D-glucosone.

Hunter and Downs (1945) have shown that non-competitive inhibition is described by the equation:

$$\frac{v}{V_i} = 1 + \frac{[I]}{K_i} \qquad (12)$$

so that the straight line obtained by plotting $[I] \cdot a/1-a$ against $[S]$ runs parallel to the $[S]$ axis.

When glucosone is pre-incubated with yeast for ten minutes prior to the addition of the glucose, results are obtained which, when analysed by either of the two methods described, show that non-competitive inhibition occurs. (Figs.X. and XV.) The K_i, calculated from Fig.XV., is 4.5×10^{-4}M, which is 50 times smaller than that obtaining in the competitive system. The Lineweaver-Burk plot (Fig.X.) shows, however, that the inhibition is not purely non-competitive, as the intercept on the 1/v axis is not increased by the same amount $(1 + I/K_i)$ as the slope of the line.

This type of inhibition has been treated theoretically by Straus and Goldstein (1943), and Goldstein (1944), and applied to the action of physostigmine, and related substances, on choline-esterase by Goldstein (1951).

2.5.5. The Zonal Analysis of Goldstein and Straus.

Straus and Goldstein (1943) pointed out that previous mathematical treatment of enzyme kinetics based on the Michaelis-Menten equation assume "that the concentration of enzyme centres is constant, and so small compared with the concentration of any substance with which it may combine that it may be neglected". They called attention to the fallacy of this assumption, and showed that enzyme concentration must be included as a factor in the mass action equation.

In considering a non-competitive system these authors showed that, at equilibrium, the relationship between enzyme [E] and inhibitor [I] can be expressed by the equation:

$$[E] + [I] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [EI] \qquad (13)$$

where the reaction $[EI] \rightarrow [E]$ + products has a negligible velocity.

Then if the mass law is followed:

$$\frac{[E - EI]\ [I - EI]}{[EI]} = \frac{k_2}{k_1} = K \qquad (14)$$

Straus and Goldstein represented the fraction of the total enzyme combined with inhibitor as "i", being equivalent to EI/E. "i" was also shown to equal $1 - v/V_{max}$. It is then possible to derive from (14) the equation:

$$I = \frac{K_i}{1-i} + iE \qquad (15)$$

which states that the total amount of inhibitor is equal to the sum of the free inhibitor and the combined inhibitor. The term E' was provided, being the "specific concentration" of the enzyme, equal to E/K, so that enzyme amount could be equated against substrate concentration.

The conditions obtaining when the enzyme concentration was very small, and when it was very large were then considered, and equations were given which described the cases where:

(a) The inhibition is a function of specific concentration of the inhibitor alone, being independent of enzyme concentration;

(b) the specific concentration of the enzyme E' is small, with practically all the inhibitor free;

(c) E' is large and practically all the inhibitor is combined.

These three forms of (15) represent three zones of enzyme behaviour, the boundaries of which are defined by the equations governing conditions (a), (b), and (c).

Michaelis and Menten (1913), Haldane (1930) and Lineweaver and Burk (1934) have based their treatment of enzyme kinetics on the assumption that the concentration of enzyme centres is so small, compared with any substance with which it may combine, that it may be neglected. From theoretical grounds, therefore, their discussions have been limited to zone A behaviour. It is also possible to show from existing experimental evidence that most systems do fall into zone A, for Haldane (1930) gives a list of dissociation constants which in most cases are greater than 10^{-5}, and as most enzyme reactions are carried out in very dilute solutions, the value of E' is bound to be less than 0.1, the limiting value calculated for zone A.

When K is small, however, i.e. when k_1 is greater than k_2 (equation 14) it is possible for E' to fall between 0.1 and 100, when zone B conditions hold; on the other hand $[E]$ may be large, and E' become greater than 100, when the system falls into zone C.

A knowledge of the value of E' will thus indicate the zone in which the system lies. Experimentally, this is accomplished by plotting "i", the fractional inhibition, against the logarithm of the specific inhibitor concentration, I', and calculating the slope of the curve at a point, $i = 0.5$. Provided that the value of the slope falls between 0.575 and 1.151, which are the limiting values for

$E' = 0.1$ and $E' = 100$, it is possible to calculate the value of E'. Easson and Stedman (1936) previously calculated $[E]$ for the choline-esterase system in the presence of physostigmine, and related substances, by another method. Dividing (15) through by "i", they obtained:

$$\frac{[I]}{i} = \frac{K}{1 - i} + [E] \qquad (16)$$

so that a plot of I/i against (1 - i) gave a straight ine with slope K and intercept $[E]$.

Goldstein (1944) adapted this type of analysis to competitive inhibition. Two equations are considered in this case:

$$\frac{[E]\,[I]}{[EI]} = K_i, \qquad (17)$$

and

$$\frac{[E]\,[S]}{[ES]} = K_s \qquad (18)$$

In order to equate enzyme concentration against inhibitor concentration, the term E'_i was provided, being defined as the specific concentration of the enzyme in terms of the inhibitor, by definition being equal to $[E]/K_i$. S' the specific concentration of substrate was defined as being equal to $[S]/K_s$. The fractional activity, "a" was considered to be equal to $[ES]/[E]$, "since enzyme activity is directly observed by measuring the rate of destruction of substrate, which rate is proportional to the concentration

of the complex $[ES]$. Term "a" is therefore equal to v/V_{max}.

From reasoning similar to that used by Straus and Goldstein (1943), three zones of enzyme behaviour are postulated. The behaviour of competitive enzyme inhibitor systems being dependent on the concentration of both substrate and inhibitor, theoretical equations must be provided which describe enzymes working in zone A with respect to substrate (E/K_s is less than 0.1) but in other zones with respect to the inhibitor; and similarly in zones B and C with respect to the substrate and other zones with respect to inhibitor.

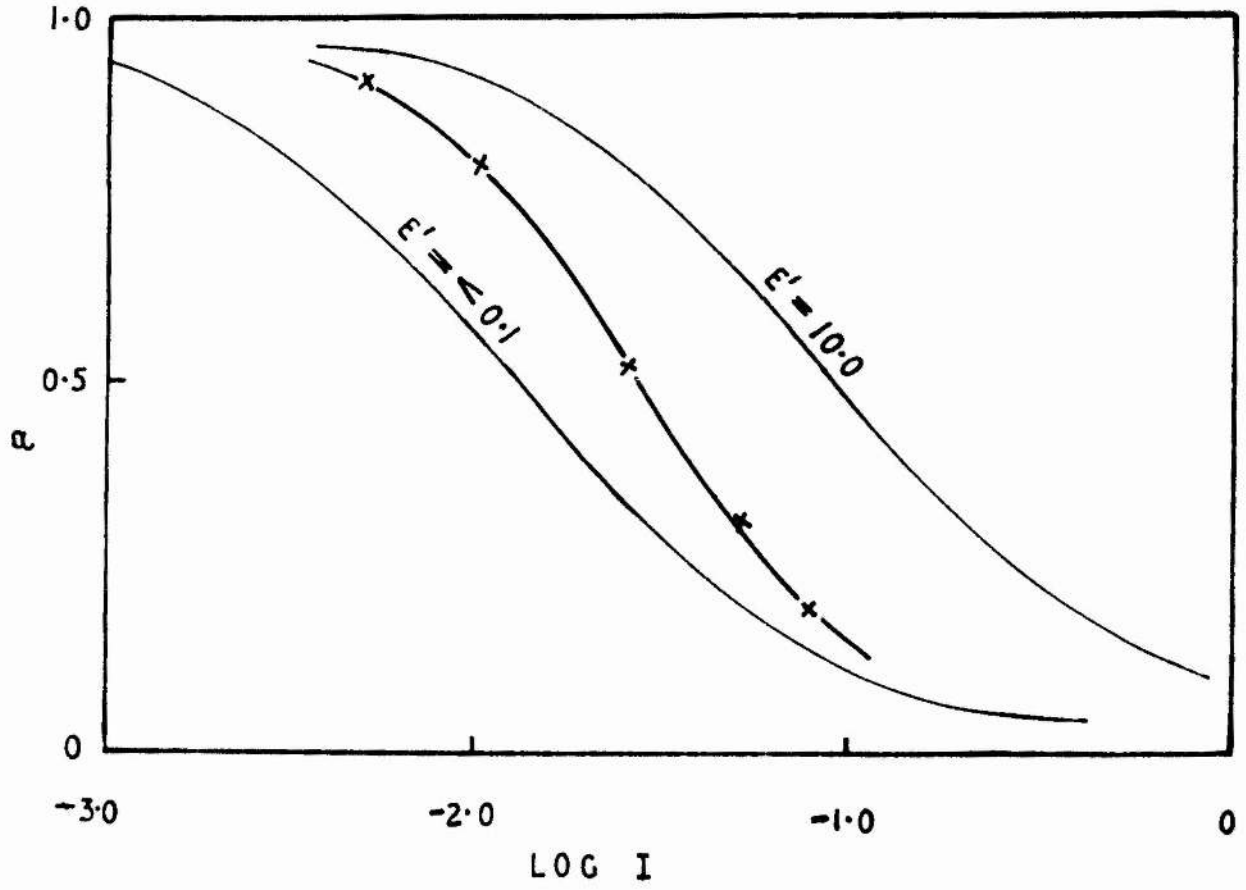
Goldstein (1944) points out, however, that competitive inhibition cannot exist in zone C, for if all of the substrate is combined with enzyme then no equation including inhibitor can be written, or conversely if S' is very small, "a" is limited to infinitesimal values.

The equation,

$$I' = S' . \frac{1-a}{a} + (1-a)E'_i \qquad (19)$$

was derived for a system where zone A behaviour was shown with respect to substrate, and zone B with respect to inhibitor. It follows from (19) that in zone A where E'_i/S is less than 0.1:

Fig.XVI. The fractional activity of glucose fermentation at varying concentrations of D-glucosone.



The labelled curves are theoretical for conditions when E , the specific concentration of enzyme, is less than 0.1, and when E is equal to 10.0. a, fractional activity of yeast fermentation; I, molar inhibitor concentration; ×—×, fractional activity at 0.01M D-glucose.

For explanation see text.

$$I' = S'. \frac{1-a}{a} \qquad (20)$$

can be written as an approximation, which is

$$\frac{I}{K_i} = \frac{S}{K_S} . \frac{1-a}{a} \qquad (21)$$

in its expanded form.

Fig.XVI. shows the fractional activity of yeast fermentation as a function of the $\log_{10}$ of D-glucosone concentration. "a", the fractional activity, is the ratio of the inhibited rate to the uninhibited rate for the period 10-40 minutes of the fermentation. The experimental curve lies to the right of the limiting zone A curve (where E' is less than 0.1) but well inside zone B (where E' varies from 0.1 to 100). The value of E'_i at a = 0.5, can be calculated as 3.58, which gives [E], the molar concentration of enzyme centres, as 2.68×10^{-2}.

Goldstein (1944) also considered systems in which "n" molecules of a substance combined with each molecule of enzyme. It was found that the slope of the a/log.[I] plot at a - 0.5 varied with "n". Thus at $n = \frac{1}{2}$, i.e. when two molecules of enzyme combined with one molecule of inhibitor, the slope varied from 0.288-1.151; where n= 1, the slope varied from 0.575-1.151; and at n = 2, the slope was 1.151 in all zones. The slope of the experimental curve of

Fig.XVI. being 0.76, it is probable that only one molecule of inhibitor combines with each molecule of enzyme under the conditions of the present experiments.

2.5.6. Pseudo Irreversible Inhibition.

The differentiation of enzyme-inhibitors into three broad categories by Straus and Goldstein (1943), and Goldstein (1944), was supplemented to some extent, by Ackermann and Potter (1949).

These authors emphasised the fact that with some inhibitors the degree of inhibition is a function of the enzyme concentration, and the situation was described where an inhibitor effectively titrated or stoichiometrically combined with an enzyme.

This phenomenon can be due to an irreversible combination of enzyme and inhibitor, or to a reversible combination of enzyme and inhibitor in which the enzyme-inhibitor complex has an extremely small dissociation constant. Ackermann and Potter (1948) refer to this latter type of inhibition as being pseudo-irreversible.

An expression was derived from the general equation used by Michaelis and Menten, but including enzyme concentration and uncombined substrate and inhibitor concentrations as further variables. In the absence of inhibitor

$$V = k \left[E_t\right] \qquad (22)$$

where V is the velocity of reaction, equal to the product of the concentration of the enzyme and its velocity constant with respect to substrate.

This expression is the equation of a straight line, k, passing through the origin, and is experimentally confirmed when values of V are plotted against corresponding values of $[E_i]$ in the absence of inhibitor (Fig.VII.). The slope is the velocity constant for the conversion of enzyme-substrate complex to product.

Equation (22) approaches

$$V = k\,[E_t] - k[I_t] \qquad (23)$$

however, as the enzyme-inhibitor dissociation constant approaches zero. That is, where the binding of the inhibitor with the enzyme is great compared with that of the substrate, i.e. the enzyme-inhibitor dissociation is very low, the equation approaches a straight line with a slope k, and an intercept on the enzyme axis. An inhibitor of this type virtually "titrates" the enzyme. Thus, the curve for an inhibitor having a dissociation constant similar to that of the enzyme-substrate complex is a straight line through the origin, and the curves for inhibitors having decreasing dissociation constants approach the limiting curve obtained with irreversible inhibitors.

The effect produced by D-glucosone on glucose fermentation with varying yeast concentration is shown in Fig.VII. (p.70) where fermentation rate is plotted against enzyme amount. It can be seen that the curve obtained by plotting inhibited rates, intercepts the abscissa at 1.2mg. (wet wt.). As no real measurement of the actual enzyme amount can be made it is not possible to calculate the real amount of enzyme titrated by the osone.

The inhibition produced by a truly competitive inhibitor is, by definition, maximal in a very short time. A state of equilibrium is quickly attained independent of the order of addition of substrate and inhibitor. Potter and Du Bois (1943) have shown that the inhibition produced by malonate in the succinoxidase system is of this type, but that the inhibition produced by copper in this system is entirely dependent on the order of addition of inhibitor and substrate. Later, Ackermann and Potter (1949) demonstrated that the irreversible inhibition produced by addition of copper to the system increases slowly with time, but the addition of substrate prevents any further increase in the amount of inhibition. The activity of the system, is at this point, proportional to the amount of enzyme which is not combined with copper. They suggest that this effect can be used to study competition between substrates and irreversible inhibitors. It must also be possible to apply

this type of test to inhibitors which have very low enzyme-inhibitor dissociation constants approaching that of an irreversible inhibitor.

The effect produced when D-glucosone is pre-incubated for varying lengths of time prior to the addition of substrate is shown in Fig.VIII. (p.83). The curve formed when the rate of fermentation is plotted against the time of pre-incubation of the inhibitor with the enzyme shows that equilibrium is not reached until the enzyme-inhibitor pre-incubation time approaches 50 minutes. Further tests showed that for times up to 4 hours there was no significantly greater inhibition of fermentation.

The effect of adding substrate to this system is to interfere with the combination of inhibitor and enzyme. The osone already combined with the enzyme is removed only very slowly, as indicated by the very gradual increase in fermentation rate after the addition of substrate. D-glucosone would appear, therefore, to fall into the category of "pseudo-irreversible" inhibitors discussed by Ackermann and Potter.

2.6. Discussion.

The fermentation experiments carried out showed broadly that D-glucosone, or the products of osone metabolism compete with D-glucose for some enzyme of glycolysis. It was also shown that the competition is limited to some point prior to the breakdown of fructose diphosphate. As hexokinase is known to be the rate limiting enzyme in the Embden-Meyerhof scheme, and in consideration of the structural similarity between glucosone and glucose it is probable that the competitive effect occurs at the active centres of the hexokinase molecule. The kinetic analysis of the results obtained has given further evidence of the nature of the D-glucosone effect in whole yeast cells.

The application of the Lineweaver-Burk (1934) analysis has shown that true competition for the enzyme centres occurs between osone and substrate added simultaneously. Although this effect is transitory in nature it does show that the inhibitor and the substrate are competing for the same enzyme centres. The later non-competitive effect is a reflection of the very small value of the enzyme-inhibitor dissociation constant.

The apparent K_i, calculated from the "competitive" results, is 7.5×10^{-3}M against the calculated K_s of 6.0×10^{-3}M, but the "non-competitive" results give a K_i of 4.5×10^{-4}M. This change in K_i may be due to the

failure of stabilisation of rate until the enzyme is saturated with inhibitor. This can be seen more readily when the dissociation constants are considered as ratios of more than two velocity constants, as suggested by Briggs and Haldane (1925). (see p.94).

Considering, $E + X \longrightarrow EX$, $EX \longrightarrow E + X$ and $EX \longrightarrow E +$ products, with rate constants k_1, k_2, and k_3 respectively, where E is the enzyme and X a substrate which will combine with E, the substance X is a substrate when k_3 is not negligible, but when k_3 is much smaller than k_2 then X is a reversible inhibitor. Thus,

$$\frac{k_2 + k_3}{k_1} = K_s \text{ when X is a substrate}$$

and

$$\frac{k_2}{k_1} = K_i \text{ when X is an inhibitor.}$$

Now Straus and Goldstein (1943) point out that the rate of reactions in the presence of inhibitors is dependent on the molar ratios of inhibitor and enzyme. As a molar concentration of D-glucosone 2.5 times that of D-glucose is required to give 50 per cent. inhibition in the system used, it is probable that the rate of combination of enzyme and substrate is greater than that of enzyme and inhibitor. k_1 for glucose is, therefore, greater than k_1

for glucosone. An indication that this assumption is valid can be obtained by studying the rate at which equilibrium is attained when inhibitor is added to an equilibrated enzyme-substrate mixture. Maximal inhibition is obtained only after 30-40 minutes (Fig.IX p.74). When no inhibitor is present equilibrium is reached in 10 minutes. As D-glucosone is here acting as a true competitive inhibitor, the fall-off in fermentation rate is a measure of the rate of combination of enzyme and inhibitor.

A similar effect has been noted by Roberts (1953) in the inhibition of bacterial glutamic decarboxylase by ∝ methyl DL-glutamic acid. When the enzyme was incubated with inhibitor before addition of substrate non-competitive inhibition was observed, but with simultaneous addition of substrate and inhibitor the inhibition was seen to be competitive. It was also noted that the degree of inhibition produced by a particular concentration of inhibitor was dependent on the time of pre-incubation of inhibitor with enzyme. Relatively high concentrations of inhibitor were required to produce inhibition when substrate and inhibitor were added to the enzyme at the same time, so that, as in the present investigation, the rate of combination of inhibitor with enzyme was assumed to be relatively small.

It is apparent, that as the combination of enzyme and inhibitor becomes less reversible the value of the rate

constant, k_2, will decrease. Inhibitors which show pseudo-irreversibility by the method of Ackermann and Potter (1949) must, therefore, have an extremely low value for k_2. The fact that pseudo-irreversibility can be shown in the present system when substrate and osone are added simultaneously, although by the classical kinetic analysis competitive inhibition can be demonstrated, would suggest that this test is one which should be applied when investigating the kinetics of any enzyme reaction.

Reif and Potter (1953a) have described the pseudo-irreversible behaviour of Antimycin A in the succinoxidase system. A characteristic property of this type of inhibitor, in addition to its titrating effect, is that it can be removed from the enzyme "by adding to the system a new component that has sufficient affinity for the inhibitor to enable the added component to compete effectively for the free inhibitor and thereby facilitate the dissociation of the enzyme-inhibitor complex".

It is possible that cyanide exerts such an effect on the glucosone inhibition of yeast fermentation (Part I., 2.3.1.).

The zonal behaviour of D-glucosone in relation to the whole yeast cell shows that the effect of enzyme concentration must be considered in this type of complex system if a more complete understanding of the reaction mechanism

is desired. When the fractional inhibition is plotted against log.I results are given which show that the enzyme concentration is not a negligible factor. A classical interpretation of the results obtained would show that competitive inhibition occurs, but mono-molecular reaction mechanisms for the destruction of inhibitor cannot be postulated when [EI] is appreciable and its reversible formation from [E] and [I] is not stoichiometric, for its destruction rate is dependent only on [EI] and not upon the concentrations of the original reactants.

As a relatively small value can be given to [E], the apparent enzyme-inhibitor dissociation constant K_i is probably small in the fully equilibrated system. It can be stated with confidence, therefore, that k_2, the rate of dissociation of enzyme and D-glucosone, is extremely small.

Assuming that the combination is reversible it is possible to explain some aspects of the <u>in vivo</u> effects of D-glucosone. It can be seen that in zone B the a/log. I plot has a fairly high slope value, so that over the middle part of this curve a small change of inhibitor concentration will give a large change in the inhibition produced. A graded dosage of inhibitor will, therefore, show no effect up to a certain point, and then, over a very small change in concentration, a strong inhibitory effect. Herring and Hynd (1928) do mention that the margin between the dose of

glucosone which produces symptoms, and the lethal dose is 0.2mg. per gramme body weight, a finding confirmed by Sakuma (1931). As the dose which produces the "glucosone effect" in mice is 2.4mg. per gramme body weight the predictions of the zone B curve would seem to be fulfilled if glucosone is acting in the inhibition of a specific animal enzyme.

Normally, in vivo, the molar concentration of enzyme centres is very high at certain localised points, so that there is effectively a high value of $[E]$. This will give a high value of E' placing the system in zone C, a condition which will occur in many in vivo systems provided that the enzyme-inhibitor dissociation constant is low. The dosage effect will then be considerably heightened, for the maximum inhibitory effect will be produced suddenly and over a very small change on inhibitor concentration. Reif and Potter (1953b) have pointed out that their postulated pseudo-irreversible inhibitors inhibit zone C behaviour, for in this zone there is a stoichiometric combination of enzyme and inhibitor. However, experimental results reported in the present work, show that a pseudo-irreversible effect is demonstrable by inhibitors falling into zone B. Thus, inhibitors cannot be placed in zone C purely because the "pseudo-irreversible" tests can be applied. It is also possible, on theoretical grounds, to show that inhibitors displaying zone B characteristics can have $[E]$ large enough

to give the titrating effect. The Ackermann-Potter tests must, therefore, be used in conjunction with others, to obtain a complete knowledge of the mechanism of any particular enzyme-inhibitor reaction.

PART II.

PHOSPHORYLATION BY HEXOKINASE.

CONTENTS

PART II.

1. The Properties of Fungal, Bacterial and Mammalian Hexokinases

Introduction.

Section 1 of this part of the thesis consists of a general review of the physical and enzymic properties of the enzymes which catalyse the phosphorylation of hexoses by ATP. Some stress is laid on a critical analysis of the specificity reported for various hexokinase preparations isolated from animal tissues, as conclusions have been reached in the past which would not seem to be substantiated when the results obtained are examined more thoroughly.

The results of experiments carried out by the author using partially purified yeast hexokinase are reported, and discussed in Section 2.

The following note on nomenclature is included as there is still some variation in the terminology used in the literature.

Nomenclature.

Meyerhof (1927) named the activator he obtained from yeast juice, hexokinase, and von Euler and von Adler (1935) believing they had separated a different enzyme, put forward the name heterophosphatese for this phosphorylating enzyme. This term, according to these authors, "combined the idea of a phosphatase with the idea of esterification by organic phosphate". In a review written in the next year, Quastel (1936) suggested that the name phosphorylase would be better, as the function of the enzyme was to phosphorylate at the expense of an already phosphorylated molecule.

The terms heterophosphatese and phosphorylase are not widely accepted and this type of enzyme was given the generic name "phosphokinase", by Dixon and Needham in 1940 (see Dixon and Needham, 1946). The term phosphorylase has now been applied to enzymes catalysing a different type of reaction.

Phosphokinases are those enzymes which catalyse the reaction:

$$ATP + X \longrightarrow ADP + XP,$$

by transfer of a terminal phosphate from ATP to the organic molecule.

Yeast hexokinase was the first of this group of enzymes to be discovered, and this name has been reserved for that enzyme, isolated from yeast, which will catalyse the transfer of phosphate to the hexose molecule, with the

formation of hexose-6-phosphate. The appropriate hexose contraction is now affixed to the term "-kinase" to denote substrate specificity within the group of mammalian, plant, fungal, and bacterial phosphokinases which have been described.

In this thesis the term "hexokinase" is used for the enzyme obtained from yeast, and "brain hexokinase" is the term used to denote the enzyme occurring in brain tissue. The specificity of other kinases is denoted by the appropriate affix.

1.1. Hexokinase.

1.1.1. Historical.

Early investigations established that phosphate esters were formed at some primary stage in glycolysis. Harden and Robison (1914) demonstrated that a mixture of hexose monophosphates, in addition to hexose diphosphate, are contained in yeast juices, and von Euler (1914), in an early work on the chemistry of yeast and alcoholic fermentation, indicated that glucose was phosphorylated by a yeast enzyme.

During the next two decades more knowledge was gained of the changes which occurred in the glucose molecule during breakdown, but little was known of the sequence of steps which led to this breakdown. The hexose monophosphate obtained earlier was investigated by Robison (1922), who suggested that this monophosphate could be an intermediate in the formation of hexose diphosphate. Five years later, Meyerhof (1927) separated an activator from bakers' yeast which, when added to aged muscle extracts, stimulated carbon dioxide formation from glucose. This partially purified enzyme Meyerhof called hexokinase, to indicate that it initiated hexose metabolism, but he did not recognise fully its significance even six years later when he published (Meyerhof and Kiessling, 1933) a scheme of alcoholic fermentation, following the scheme of Embden, Deuticke, and Kraft (1933)

for lactic acid fermentation in muscle extracts. Both of these schemes used hexose diphosphate as their starting point, although Meyerhof and Kiessling (1933) considered that the primary ester formed in muscle glycolysis differed from the hexose or triose phosphates then known.

Neuberg (1935) indicated that the addition of fructose monophosphate and adenylic acid to a fresh yeast suspension led to the formation of hexose diphosphate and ATP. At this time, Schaffner, Bauer, and Berl (1935) reported that the phosphorylation of glucose by "yeast phosphatese" required the presence of the hexosephosphate-dehydrogenase system, this system being a complex mixture of DPN, ATP, Mg^{++}, hexose monophosphate, and a dehydrogenase. In a later paper Schaffner, Berl, and Bauer (1935) demonstrated that the purification of yeast maceration juice removed an enzyme necessary for the conversion of hexose monophosphate to hexose diphosphate.

It was left to von Euler and von Adler (1935), and Lutwak-Mann, and Mann (1935) to demonstrate that an enzyme from yeast activated the transfer of phosphate to glucose with the formation of hexose monophosphate. von Euler and von Adler (1935) indicated that Mg^{++} was necessary for full activity of this enzyme, and later Ohlmeyer and Ochoa (1937) showed that Mn^{++} could also act as an activator.

The role of ATP in transphosphorylation was also elucidated at this time. Lohmann (1929) and Fiske and SubbaRow (1929) almost simultaneously isolated a crystalline silver salt of ATP, and suggested that ATP itself was a normal constituent of muscle. Later Lohmann (1931) stated that this compound was necessary for the glycolytic activity of muscle extracts.

Meyerhof and Lohmann (1931) were the first to suggest that ATP was concerned in the primary esterification of carbohydrate, labile phosphoric acid groups being transferred to the carbohydrate during muscle glycolysis, the ATP being resynthesised during a later stage in glycolysis. In a further investigation it was shown by Lohmann (1934) that creatine phosphate wassplit by muscle extracts in the presence of adenylic acid, to form ATP, and in the next year the reverse of this reaction, the phosphorylation of creatine by ATP was demonstrated by Meyerhof and Lohmann (1935) and Needham and van Heyningen (1935).

Thus the role of ATP as a transphosphorylating coenzyme was established, and Meyerhof (1935) suggested that the initiating reaction in glycolysis in yeast and in muscle was the phosphorylation of hexoses by ATP, the phosphate being transferred according to the equation:

ATP + 2 glucose → adenylic acid + 2 glucose-6-phosphate.

An electrophoretic study of the hexokinase of Meyerhof, and the heterophosphatese of von Euler, by Meyerhof and Mohle (1937) demonstrated that these two fractions from plasmolysed yeast and yeast maceration juice, were identical. When Colowick and Kalckar (1941) demonstrated that, in yeast, only one phosphate group was transferred from ATP to glucose and fructose, and not two as previously suggested, the catalytic action of hexokinase could be defined as the transference of phosphate from ATP to the hexose molecule according to the equation:

ATP + hexose → adenosine diphosphate + hexose-6-phosphate.

Hexokinase was later brought into prominence on account of its high specific sensitivity in the body. The high sensitivity of the enzyme to vesicants, like mustard gas, was discovered in 1940 by Dixon and Needham (1946), who pointed out a parallelism in the hexokinase inhibition and the vesicant properties of nitrogen mustards. The interest stimulated by this observation led to the crystallisation of the enzyme by three groups of workers. (Bailey and Webb, 1948; Berger, Slein, Colowick, and Cori, 1946; and Kunitz and MacDonald, 1946).

1.1.2. Physical Properties.

The crystalline enzyme is shown by electrophoretic, solubility, and ultracentrifuge studies to be a pure, single protein. The molecular weight is 96,600, at pH 5.5 and 1°, from sedimentation and diffusion studies. The enzyme has a maximum stability at pH 4.5 to 4.8, the iso-electric point.

1.1.3. Specificity.

Kunitz and MacDonald (1946) showed that the pure enzyme acted on D-glucose, D-fructose, and D-mannose, and not on D-galactose, L-arabinose, D-xylose, L-rhamnose, sucrose, maltose, lactose, trehalose, or raffinose.

Slein, Cori, and Cori, (1950) have shown that competition can be demonstrated between D-glucose, D-fructose and D-mannose for hexokinase, which suggests that there is a common active centre for the activation of all three hexoses.

The phosphorylation of D-glucosamine by ATP using crystalline yeast hexokinase has been described by Brown (1951) and by Grant and Long (1952). Cramer and Woodward (1952), using a partially purified yeast hexokinase, have shown that 2-deoxy glucose is phosphorylated, and Johnstone and Mitchell (1953) also using a partially purified hexokinase found that D-glucosone is phosphorylated at about the same rate as glucose.

All of the evidence so far presented concerning the phosphorylation of sugars by hexokinase points to the fact that one single enzyme is present, able to catalyse the phosphorylation of several sugars having closely related structures.

1.1.4. Kinetics.

In general, the relative rates of phosphorylation of the different substrates depend on the sugar concentration. Glucose is attacked more rapidly than fructose at low hexose concentrations, while fructose is attacked more rapidly at high sugar concentrations. Dixon and Needham (1946) pointed out that this indicated a relatively low fructose affinity for the enzyme, an observation borne out by later quantitative determinations.

From studies with whole yeast cells Gottschalk (1945, 1947), concluded that only the furanose form of fructose can be phosphorylated by yeast hexokinase. In agreement with these findings Slein, Cori, and Cori (1950) showed that, at pH 5.9 at 0^{o}, the rate of D-fructose utilisation by crystalline hexokinase was limited by some non-enzymic factor, as glucose utilisation was not limited under these conditions. They also showed that the rate of phosphorylation of freshly dissolved D-fructopyranose was significantly lower than that of an equilibrated solution of fructose.

The dissociation constants of the enzyme-substrate complexes have been quoted in only a few cases. vanHeyningen, as quoted by Dixon (1941), gives a value of 1×10^{-3}M for the glucose-enzyme complex, and 2×10^{-3}M for the fructose-enzyme complex. In a later communication Slein (1950) reported the following values for the substrate-enzyme complex: glucose, 1.5×10^{-4}M; mannose, 1.0×10^{-4}M; and fructose, 1.5×10^{-3}M. Wajzer (1953) stated that the Michaelis constants for glucose and fructose were similar, being of the order of 5×10^{-4}M. He also found that fructose was phosphorylated at about half the rate of glucose when an equimolar mixture of the sugars was used.

The turnover number for the crystalline enzyme at pH 7.5 and 30°, is 13,000 mols. of glucose/mol. of enzyme/min. (Berger, et al, 1946). When saturated with mannose the value is 6,500 and when saturated with fructose is 26,000.

Brown (1951) states that the turnover rate of glucosamine is 12,000 mols./mol. of hexokinase/ minute at pH 7.8 at 30°; glucosamine is phosphorylated at about 70% of the rate of glucose according to Grant and Long (1952). Dixon and Needham (1946) have shown that the enzyme has a broad pH optimum between 8 and 9.

1.1.5. Activation.

von Euler and von Adler (1935) found that hexokinase was strongly activated by Mg^{++} ions, a fact confirmed by Berger et al (1946) who give a dissociation constant of 2.6×10^{-3}M for the Mg-enzyme complex. Mn^{++} ions also activate hexokinase according to von Euler, Adler, and Vestrin (1937) and Ohlmeyer and Ochoa (1937) but no details concerning the dissociation constant of the Mn-enzyme complex are known.

A specific protein activator has been extracted from muscle by Weil-Malherbe (1951) which causes a two to five-fold increase in hexokinase activity. The activator is not associated with any other enzyme and is specific for the phosphorylation of glucose and fructose. It is not known in what manner this protein activates the hexokinase, but the non-specific stabilising action of insulin and other proteins has been described by Berger et al (1946).

1.1.6. Inactivation.

van Heyningen (1942) investigated the mode of action of mustard gas and lachrymators on hexokinase during the period 1940 to 1941 and found hexokinase to be an enzyme containing -SH groups: it was, therefore, inactivated by oxidation or by alkylating agents such as ethyl iodoacetate. The enzyme was also inactivated by mustard gas by reason of a combination of the inhibitor with some group

or groups necessary for enzyme action, about 6 molecules of mustard gas being attached to each molecule of protein according to Bailey and Webb (1948), and Harriott, Anson, and Northrop (1946).

On account of similarity between ethyl iodoacetate and iodoacetic acid, and the latter's well-known inhibitory action on -SH enzymes (Quastel, 1933; Dickens, 1933), the effects of the lachrymators on these enzymes were investigated by many groups of workers between 1940 and 1942.

van Heyningen showed that the -SH form of hexokinase was completely inhibited by 1.2×10^{-3}M ethyl iodoacetate but the oxidised form was not inactivated. The monothiol lewisite, $ClCH=CHAsCl_2$, protected hexokinase to some extent from the action of ethyl iodoacetate, this being evidence for the presence of -SH groups in the enzyme, as lewisite is known to combine with these -SH groups.

Berger *et al* (1946) reported that neither cysteine nor glutathione exerted any stimulatory or protective effect on hexokinase, and concluded that -SH groups were not essential for enzymic activity. Bailey and Webb (1948) later demonstrated that crystalline hexokinase is completely inactivated by 0.016M sodium iodoacetate, confirming the results of van Heyningen (1942) with a partially purified preparation of the enzyme.

It was shown by Kunitz and MacDonald (1946) that hexokinase was inactivated rapidly by trypsin, but not by chymotrypsin. The proteolytic enzymes present in crude yeast plasmolysates also inactivate hexokinase very rapidly. van Heyningen (1942), during her early work on the enzyme, noted that glucose had a strong protective action against this proteolytic effect, fructose being much less effective. The report of this protective effect was one of the factors which later allowed the crystallisation of the pure enzyme. Berger et al (1946) reported that fructose and glucose gave complete protection against the loss of fructose and glucose phosphorylation activities, respectively, but did not check whether fructose afforded protection against the loss of glucose phosphorylating activity. They also reported that glucose protected the pure enzyme against inactivation by trypsin. The gradual inactivation of solutions of the pure enzyme can be prevented, according to Kunitz and MacDonald (1946), by glucose or mannose, and to a much smaller extent, by fructose or sucrose.

They also found that dilute hexokinase solutions were stabilised by 1% glycine about 70% as effectively as by 1% glucose. The present author has confirmed this stabilising effect of glycine and used it in the isolation of a partially purified hexokinase.

The spontaneous inactivation of solutions of the pure enzyme can also be prevented by dilution in the presence of proteins, insulin in a concentration of 10g./ml. being found very effective by Berger et al (1946).

Although fluoride is known to be a potent inhibitor of Mg^{++}-containing enzymes, on account of its Mg-binding properties, Berger et al (1946) reported that hexokinase was unaffected by fluoride up to concentrations of 0.125M, when the Mg^{++} concentration was 6.5×10^{-3}M and the orthophosphate concentration 1×10^{-3}M. Bailey and Webb (1948) on the other hand, report a 46% inactivation of hexokinase activity by 0.04M sodium fluoride in the presence of 1.1×10^{-3}M $MgCl_2$, and Wagner and Yourke (1953) state that 0.15-0.20M sodium fluoride inhibits hexokinase activity completely. Warburg and Christian (1942) showed that the inhibition of enolase by fluoride depends on the presence of orthophosphate. As Berger et al (1946) were the only investigators to specify the orthophosphate concentration used, the differences in the effect of fluoride on hexokinase reported by the three groups are possibly due to variations in the concentrations of phosphate present.

Colowick and Kalckar (1943) noted that Robison's ester does not inhibit the phosphorylation of hexose by ATP and later Slein, Cori, and Cori (1950) reported that glucose-6-phosphate did not inhibit crystalline yeast hexokinase.

Weil-Malherbe, and Bone (1951) also reported that glucose-6-phosphate did not inhibit a partially purified hexokinase preparation. More recently, Crane and Sols (1953) indicated that the activity of the pure enzyme was reduced by 9 per cent. in the presence of 0.01M glucose-6-phosphate. Wajzer (1953) using the fraction of yeast maceration juice precipitated between 50 and 75 per cent. saturation with ammonium sulphate, has stated that glucose-6-phosphate inhibits the phosphorylation of glucose. He also suggested that glucose-6-phosphate was inhibitory towards fructose phosphorylation in a competitive manner. It is difficult, however, to accept the validity of the conclusions reached from the experiments described. Wajzer (1953) used a preparation of Embden's ester as the source of the inhibitory glucose-6-phosphate so that although fructose-6-phosphate was used as a control, and showed no inhibitory effect towards either glucose or fructose phosphorylation, it cannot be at all certain that the hexokinase reaction is the only one being measured, or that the addition of hexose monophosphates do not make the ATP rate-limiting in this complex system. The hexokinase reaction can be considered to be irreversible, as there is a loss of about 8,000 calories per molecule of glucose-6-phosphate formed (ATP has a high phosphate bond energy of about 10,000 to 12,000 calories, while that of glucose-6-phosphate is about 3,000 calories per molecule). Thus,

if glucose-6-phosphate were inhibitory towards the phosphorylation of glucose it would indicate a means whereby the amount of glucose metabolised by the cell could be controlled, for the rate of initial glucose phosphorylation would depend on the rate of the further metabolism of glucose-6-phosphate.

It has been reported by Slein, Cori, and Cori (1950) that glucose-1-phosphate, glucose-6-phosphate, mannose-6-phosphate, and galactose-1-phosphate at concentrations of 10^{-3}M did not inhibit fructose utilisation by yeast hexokinase, and also that glucose phosphorylation was not inhibited by mannose-6-phosphate.

The adenine nucleotides do not seem to have any regulatory effect on this enzyme, as it was reported by Colowick and Kalckar (1943) that adenosine diphosphate and adenylic acid were not inhibitory towards hexokinase activity, a finding confirmed by Weil-Malherbe and Bone (1951) with a similar partially purified hexokinase preparation.

The activity of partially purified hexokinase is not affected by DL-glyceraldehyde (Adler, Calvet, and Günther, 1937; Lardy, Wiebelhaus, and Mann, 1950) although glucose fermentation by dried yeast extracts is inhibited (Adler et al, 1937; Boyland and Boyland, 1938). Lardy et al, (1950) have also reported that L-sorbose-1-phosphate had no inhibitory effect on partially purified hexokinase.

1.1.7. Conclusions.

Present evidence suggests that hexokinase is not specific for the common fermentable sugars. Certain small variations of C2 of the glucose molecule are permissible without affecting the phosphorylating activity of the enzyme.

It has been shown that glucose is phosphorylated in position 6 (Colowick and Kalckar, 1943) as is mannose (Slein, 1950), and glucosamine (Brown, 1951); Colowick and Kalckar, (1943) demonstrated by an indirect method, using partially purified hexokinase, that fructose is phosphorylated at position 6. More recently, Slein et al (1950), using crystalline hexokinase, confirmed Gottschalk's (1945, 1947) assumption that the exclusive fermentation of the furanose form of fructose by yeast was due to the specificity of hexokinase for this form of the sugar. They stated that "the enzyme reacts with that form of fructose in solution which contains a free hydroxyl group on C6". The structures of the phosphorylated derivatives of 2-deoxy glucose and glucosone have not been elucidated but it is probable that a 6-phosphate is formed in each case.

It would appear that hexokinase is not inhibited by its reaction products. Although the effect of many natural sugar phosphates on hexokinase has been investigated, the effect of fructose-1-phopphate, formed in some animal tissues, has not been reported.

As further indications have recently been obtained of the specificity of hexokinase, by the use of partially purified preparations of the enzyme, a more complete investigation using pure hexokinase is now justified.

1.2. Mammalian Hexokinases.

The mammalian hexokinases differ in some respects from yeast hexokinase but also appear to have some similar properties. They are considered here in some detail in order that an indication might be obtained of the effect which could be produced by substances known to affect yeast hexokinase.

1.2.1. Historical.

Although evidence for the presence of hexokinases in animal tissues was not presented until several years after yeast hexokinase had been established as a definite entity, much presumptive evidence had accumulated which made fairly certain that a similar enzyme did exist. Meyerhof (1927) showed that hexose phosphates were formed during the fermentation of glucose by fresh muscle extracts, and later with Lohmann (1931) presented evidence which led them to suggest that ATP acted as a phosphate donor in muscle glycolysis.

The later studies made by Dische (1935) on lysed red blood cells demonstrated that the breakdown of glucose or hexose monophosphate was preceded by a phosphorylation occurring through the mediation of ATP. Many more indirect observations made during the next five years indicated that glucose was phosphorylated by a hexokinase similar to that found in yeast, but the first direct observation of hexokinase

activity in animal tissues was made by Geiger (1940) using brain extracts. Colowick, Kalckar, and Cori (1941) were unable to demonstrate this direct transfer of phosphate to glucose in mammalian tissues but Ochoa (1941) was able to confirm Geiger's (1940) findings.

Ochoa (1941) introduced at this time the technique of studying the reaction with acetone-dried tissue extracts in the presence of potassium fluoride which acted as a stabiliser of the hexokinase and an inhibitor of the ATPase. Using this technique Cori, Colowick, Berger, and Slein (1941-44) were able to demonstrate the presence of hexokinase in retina, brain, heart, liver, kidney and skeletal muscle.

There was at this time no evidence to show that more than one enzyme was responsible for the phosphorylation of all the sugars tested, but later experiments have shown that specific glucokinases and fructokinases are present in various tissues.

1.2.2. Physical Properties.

The physical properties of the mammalian hexokinases are unknown as none have so far been obtained in a pure crystalline state.

1.2.3. Specificity.

The rate of glycolysis of glucose and fructose is different in various tissues. This difference may be due

to the existance of separate hexokinases which activate glucose or fructose respectively, or to a single enzyme which reacts with glucose much more readily than with fructose.

Many of the results obtained are open to suspicion, however, as relatively impure enzyme preparations are being used, and there is a lack of adequate control experiments. The results reported regarding the specificity of brain, liver and muscle kinases will be considered here, in that order. The absorption of hexoses into the intestine is also discussed, at the end of this section, as it is generally considered that such absorption occurs through a phosphorylating mechanism.

It was demonstrated by Meyerhof and Geliazkowa (1947) that in brain and tumour tissue, glucose is metabolised much faster than fructose at low sugar concentrations, in confirmation of the findings of Boyland and Boyland (1938). Meyerhof (1947) suggested that this effect might be due to a much lower affinity of fructose for brain hexokinase than of glucose for the enzyme, at low ATP concentrations. In the next year Meyerhof and Wilson (1948), using brain homogenates and crude extracts, reported that at low hexose concentrations, fructose had a much lower affinity for brain hexokinase than did glucose. In the presence of 8×10^{-4}M potassium naphthoquinone sulphonate and at low hexose concentrations,

the rate of fructose phosphorylation was shown to be lower than that of glucose, but in the presence of the inhibitor at high hexose concentrations the rates were similar. As, according to these authors, yeast shows no difference in affinity for glucose or fructose at low hexose or ATP concentrations, it was suggested that two separate kinases are present in brain tissue.

An acetone-dried beef brain powder was used by Harpur and Quastel (1949) in a study of the phosphorylation of glucose, fructose and glucosamine, phosphorylation being measured by the method of Colowick and Kalckar (1943) in the presence of iodoacetate. The results obtained purported to describe mutual competition between the substrates for a phosphorylase, but as the experiments were carried out at a rate-limiting ATP concentration, the conclusions reached need not be the correct ones. The inhibitory effect of N-acetyl glucosamine on the phosphorylating of glucose, fructose and glucosamine in this complex system, does no more than suggest that one enzyme may be involved in the phosphorylation of all three substrates, and does not as stated by the authors "make it difficult to believe that is not the case".

Wiebelhaus and Lardy (1949) also used acetone-dried beef brain to demonstrate a higher rate of phosphorylation of glucose than of fructose. This system phosphorylated D-glucose-, D-fructose-, and D-mannose- 6 phosphates but

not L-glucose, D-galactose, L-sorbose, D-ribose, D-arabinose, L-rhamnose, D-xylose, potassium D-gluconate or potassium 2-oxo-D-gluconate. It was found that sodium ions were inhibitory towards glucokinase activity to some extent in a dialysed, lyophilised beef brain preparation; the phosphorylation of D-fructose or D-fructose-6-phosphate was not affected. In addition, certain extracts were found to be more active towards glucose, whereas other were obtained having greater fructokinase activity.

Considered together, these results (Meyerhof and Wilson, 1948; Harpur and Quastel, 1949; Wielbelhaus and Lardy, 1949) indicate that two kinases having different substrate affinities may exist in brain tissue. The sodium effect has not been confirmed, however, and the difference between the phosphorylation rates of glucose and fructose may not be significant when the type of enzyme preparation is considered.

The phosphorylation of D-fructose by brain hexokinase is strongly inhibited by D-glucose and D-mannose, according to Slein, Cori, and Cori (1950), there being only 15 per cent. of normal fructose utilisation when the initial concentration of fructose was four times that of glucose. As fractionation of the enzyme preparation with ammonium sulphate or acetone failed to produce a shift in the ratio of glucose to fructose utilised, it was considered that the

"predominant type of hexokinase found in an extract of acetone-dried brain is of the yeast type". Iso-electric precipitates of the brain enzyme gave"somewhat lower F:G ratios" however, so that these results could also be interpreted as being proof of the existence of separate phosphorylating enzymes for glucose and fructose.

All of the kinase preparations used in the work mentioned were known to be complex mixtures of enzymes so that the results obtained are open to many interpretations. A much purer brain hexokinase preparation was obtained by Crane and Sols (1953) by treating certain particulate material from calf brain with lipase and sodium deoxycholeate. The material obtained on high speed centrifugation of the treated preparation had high hexokinase activity and no phosphofructokinase activity. It was stated that this preparation phosphorylated glucose, fructose, 2-deoxy glucose and mannose, and there was found to be no difference between the rates of phosphorylation of glucose and fructose at low substrate concentrations. These results are not evidence that enzymes specific for glucose or fructose do not exist in brain tissue, although Crane and Sols concluded that the presence of a single enzyme was indicated, having a specificity similar to that of yeast hexokinase.

Specific kinases have been identified more definitely in liver and muscle tissue. Cori (1941) mentioned the

isolation of fructose-1-phosphate from rat liver homogenates and six years later Cori and Slein (1947) stated that glucose and fructose are phosphorylated by different enzymes in liver tissue. Vestling, Irish, Hirsch, and Grant (1949) stated that a fructokinase, having no glucokinase activity, had been separated from liver tissue, and in a later paper Vestling Mylroie, Irish, and Grant (1950) showed that a rat liver extract phosphorylated fructose under aerobic conditions at ten times the rate of glucose.

A fructokinase was definitely obtained from liver by Leuthardt and Testa (1950a) and shown to phosphorylate D-sorbose and D-tagatose in addition to fructose, but not mannose, glucose, or galactose. These authors suggested the name ketohexokinase for the enzyme.

The existence of an enzyme which specifically phosphorylated galactose was postulated by Kosterlitz (1943), who had previously (Kosterlitz, 1937) shown that the administration of galactose to rabbits produced an accumulation of galactose-1-phosphate in the liver. It was found that the treatment of liver extracts with bentonite gave kinase preparations having much greater galactokinase activity than glucokinase activity (Trucco, Caputto, Leloir and Mittelmann, 1948) and fractionation with acetone or alcohol gave preparations having higher glucokinase activity. The

existence of galactokinase as an enzyme specific for galactose phosphorylation was demonstrated finally by Bacila (1949), who obtained a galactokinase preparation from liver which showed no glucokinase or fructokinase activity. Leloir and Cardini (1953) mentioned that a liver galactokinase has been isolated which will phosphorylate D-galactosamine. This reaction is expected in view of the reported phosphorylation of glucosamine by brain hexokinase (Harpur and Quastel, 1949).

Crude extracts of muscle rabbit are able to phosphorylate fructose, glucose and mannose. Slein et al (1950) demonstrated that fractions having high glucokinase activity can be separated from thosehaving high fructokinase activity, by fractional precipitation of muscle extracts with acetone or ammonium sulphate; a definite separation of glucokinase and fructokinase from muscle extracts was reported in the next year by Cori, Ochoa, Slein, and Cori (1951). Colowick (1951a) has stated that mannose is also phosphorylated by a fraction of rat muscle which will phosphorylate glucose and fructose. As a specific fructokinase could be isolated, in addition to the kinase acting on glucose, fructose and mannose, it was concluded that there are present in animal tissues at least two hexokinases.

The process of absorption of carbohydrates into the intestine was considered to be due to phosphorylation in

the hexose-absorbing intestinal mucosa, independantly by Wilbrant and Laszt (1933) and Lundsgaard (1933). It was shown by Lundsgaard (1933) that phloridzin caused a marked and specific inhibition of phosphorylation, which agreed in theory with the work of Nagano (1902) who had demonstrated a phloridzin inhibition of intestinal glucose absorption. Later, Lastz and Süllman (1935) found increased amounts of phosphate esters present in the intestinal wall following the absorption of hexoses, a finding confirmed by Verzar and Süllman (1937) and Lundsgaard (1939). In their well-known monograph on the subject, Verzar and McDougall (1936) explained the selective absorption of certain hexoses as a phosphorylation which increased the diffusion gradient, followed by a dephosphorylation inside the cell. The esters formed in the intestinal mucosa when glucose, fructose and galactose were administered to rabbits, were finally isolated and analysed by Kjerulf-Jensen (1942) and found to be monophosphate esters. Beck (1942) also demonstrated that the amount of hexose monophosphate present in the rat intestinal mucosa increased during hexose absorption. The role of hexokinase in the intestinal absorption of sugars was even more strongly indicated by the close correlation found by Hele (1950, 1953) between the absorption rates and rates of phosphorylation of various sugars in the rat intestine. As the galactose

phosphorylating activity was very labile it was suggested that a galactokinase was present, separate from the enzyme which phosphorylated glucose, mannose and xylose. Long, (1952) has pointed out, however, that the hexokinase activity of rat intestinal mucosa is such that all of the normal carbohydrate intake, measured as glucose, cannot be phosphorylated by the mucosa.

1.2.4. The Structure of the Phosphorylated Products.

The products formed by the action of animal phosphokinases have similar structures to those obtained by the action of hexokinase. Much of the evidence obtained is of an indirect nature, as the impurity of the enzyme preparations does not allow direct observation to be made, but it has been established that fructose-1-phosphate is the product of fructose phosphorylation in liver tissue.

The primary product of glucose phosphorylation in brain extracts was shown to be glucose-6-phosphate by Colowick, Cori, and Slein (1947). It was stated later (Slein *et al*, 1950) that as they could determine the rate of fructose phosphorylation by measuring the rate of formation of fructose-6-phosphate in an enzymic system which did not affect fructose-1-phosphate, fructose was phosphorylated at C6 in brain tissue. The structures of the phosphate esters formed from other hexoses by brain hexokinase have not been reported.

The galactose ester accumulating in the liver tissue on administration of galactose was found to be galactose-1-phosphate (Kosterlitz, 1943) although no proof was given that this was the primary ester formed. Trucco, et al (1948) state that galactose-1-phosphate is formed by a liver galactokinase, however, and a similar preparation is said to phosphorylate galactosamine on C1 (Leloir and Cardini, 1953).

Fructose-1-phosphate was isolated from liver tissue by Cori (1941) and by Kjerulf-Jensen (1942) following the administration of fructose. The ester was finally identified as the product of fructose phosphorylation by liver fructokinase by Leuthardt and Testa (1950) and Cori et al (1950). The structure of the glucose ester formed on phosphorylation of glucose by liver glucokinase was not described by the latter authors but it was considered that glucose-6-phosphate may be produced.

The hexose phosphates formed by the action of muscle kinases have not yet been definitely identified.

Slein (1949, 1950) reported the existence of phosphomannose isomerase in rat and rabbit muscle and this finding was considered to be evidence for the primary phosphorylation of mannose on C6, by Colowick (1951b), but this is not a legitimate assumption in view of the crude extracts used.

Kjerulf-Jensen (1942) stated that the fructose ester accumulating in intestinal mucosa on administration of

fructose was fructose-1-phosphate, but there is no evidence that this is the primary ester formed. Glucose-6-phosphate and galactose-6-phosphate were also identified but it is not knownwhether these esters are formed directly by the action of mucosal kinases.

1.2.5. Kinetics.

The kinetic data reported by several investigators (Wiebelhaus and Lardy, 1949; Wiel-Malherbe and Bone, 1951; Slein et al, 1950) have been calculated from experiments using partially purified phosphokinases so that the data may have very little significance. The relative rates of phosphorylation of different sugars in various tissues have been quoted by some workers (Meyerhof and Wilson, 1948; Slein et al, 1950; Colowick, 1951b; Sols and Crane, 1953) but their figures are difficult to accept on account of the several hexokinases known to exist in the tissues.

It is apparent, however, that glucose has a much higher affinity than fructose for brain hexokinase, at low sugar concentrations (Meyerhof and Wilson, 1948; Weibelhaus and Lardy, 1949; Sols and Crane, 1953) but at higher sugar concentrations the rate of phosphorylation of fructose is higher than that of glucose (Harpur and Quastel, 1949; Crane and Sols, 1953). These findings could indicate the presence of either a single enzyme which activates both

glucose and fructose or of separate specific phosphokinases.

1.2.6. Activation.

Mg^{++} ions have been shown to be necessary for complete activation of muscle glucokinase and fructokinase by Slein et al (1950). Maximal hexokinase activity of various rat tissues was obtained only in the presence of 0.005M $MgCl_2$ according to Long (1952). Manganous ions, in the absence of phosphate, will substitute for magnesium ions with brain hexokinase, according to Crane and Sols (1953), but cobaltous ions appear to be ineffective. A liver homogenate which had itself no hexokinase activity, but which increased the activity of brain hexokinase was described by Vestling et al (1950). Weil-Malherbe and Bone (1951) obtained activators of rat brain hexokinase and muscle hexokinase from erythrocytes, and muscle extracts.

They suggested that the activation exhibited by the muscle and erythrocyte factor may be caused by the catalysis of the conversion hexopyranose $\rightleftharpoons$ hexofuranose, or by direct activation of the hexokinase molecule.

1.2.7. Inactivation.

1.2.7.1. Chemical.

The rapid disappearance of hexokinase activity from aqueous extracts of muscle has been reported by many investigators. This loss occurred rapidly at pH 6, but

not at all at pH 7, according to Colowick and Price (1945). Later Colowick (1947) demonstrated that a dialysable factor present in boiled muscle juice is responsible for the stability of the enzyme in alkaline media. Ochoa (1941) demonstrated that KF has a stabilising action on mammalian hexokinases; although this fact has been accepted by most investigators and used in the isolation of active hexokinase extracts it was definitely confirmed by Long (1952) using various rat tissues. Wagner and Yourke (1953) state, however, that NaF at concentrations of 0.15-0.20M inhibits the hexokinase activity of leucocytes completely. The stabilising action of Versene, cysteine and cyanide on purified brain hexokinase activity was demonstrated by Crane and Sols (1953) who also reported that amorphous (Zn-Free) insulin and crystalline serum albumin provided partial protection of the diluted enzyme.

Griffiths (1949) has suggested that muscle hexokinase has essential -SH groups, as he obtained a complete inactivation of muscle hexokinase by 5.0×10^{-3}M alloxan, which was reversed by cysteine. The inhibition of rat muscle hexokinase by iodoacetate reported by Colowick (1951a) would support the suggestion that -SH groups are essential. Weibelhaus and Lardy (1949), on the other hand, added iodoacetate to check further glycolysis in the brain extracts used as a source of hexokinase. Brain hexokinase was

inhibited by p-chloromercuribenzoate and o-iodosobenzoate according to Crane and Sols (1953) which demonstrates the presence of essential sulphydryl groups. Kun (1952) has also shown that glyoxal, a strong inhibitor of -SH enzymes, inactivated rat brain and muscle phosphokinases.

Various other inhibitors of hexokinases have also been described. Greig (1948) described an inhibition of brain glycolysis by 6-dimethyl-amino-4:4-diphenylheptan-3-one hydrochloride (amidone). Hexokinase was considered to be the site of this inhibition, as the further breakdown of glycogen, fructose-6-phosphate and hexosediphosphate were unaffected. The hexokinase activity of rat tumour tissue is unaffected by amidone, according to Boyland, Goss and Williams-Ashman (1951), and this finding is probably more acceptable than that of Greig using indirect methods.

Meyerhof and Wilson (1948) obtained an inhibition of brain extract hexokinase with saturated octyl alcohol and saturated phenylurethane, although normally these substances inhibit by absorption onto cell surfaces. In a later paper Meyerhof and Randall (1948) described an inhibition of brain hexokinase by adrenochrome, and by o-naphthoquinone. The inhibitions were not due to any limitations of ATP, as excess ATP was measured at the end of the experiments carried out. The inhibition of brain hexokinase by these various narcotic substances, if found to be specific, will account

for some of the properties of this group of substances.

Boyland et al (1951) tested the effect of a number of mitotic poisons on hexokinase activity, and found that only methyl bis(2-chloroethyl)-amine had any significant effect. They conclude that the inhibition of hexokinase is not an essential characteristic of mitotic poisons.

They also found that 0.009M phloridzin had no inhibitory effect on tumour hexokinase with glucose as substrate, although Lundsgaard (1933) showed that phloridzin was a specific inhibitor of phosphorylation by intestinal mucosa.

Racker and Krimsky (1946) reported that the glycolysis of glucose by mouse brain extracts was strongly inhibited by Na^+ ions. Since sodium ions did not inhibit lactate formation from either glucose-6-phosphate or fructose-6-phosphate they suggested that the inhibitory effect was exerted at the primary stage of hexose phosphorylation. Sodium ions inhibit the glucokinase activity of dialysed beef brain according to Weibelhaus and Lardy (1949), but fructose phosphorylation is unaffected. Long (1952) confirmed that Na^+ ions produce an inhibition of the hexokinase activity of rat intestinal mucosa but the inhibition found was much less than that described by Weibelhaus and Lardy (1949).

However, Utter (1950) was unable to produce any inhibitory effect with sodium ions up to a concentration of 0.14M. Sodium ions did stimulate ATPase, however, which

would give rise to a greater concentration of adenylic acid. This finding could possibly explain the results obtained by Racker and Krimsky (1946), for Greenberg (1949) has shown that adenylic acid is inhibitory towards glycolysis. Weil-Malherbe and Bone (1951) were unable to produce any inhibition of rat brain hexokinase activity with 0.0132M sodium ions, confirming the findings of Utter (1950).

The studies on the mutual competition between, and inhibition by substrates utilised by these enzymes have been discussed. The inhibitory effects of other substances will be considered here.

Weil-Malherbe and Bone (1951) found that D- glucose phosphorylation by brain hexokinase was strongly inhibited by hexose monophosphate at low concentrations. In the presence of isomerase, glucose-6-phosphate was found to be more inhibitory than fructose-6-phosphate, and the inhibitory effect of Embden's ester was midway between these two pure components. It was considered that glucose-6-phosphate displayed a non-competitive inhibitory effect with respect to glucose and ATP. In a recent communication, Sols and Crane (1953) confirmed this non-competitive inhibitory effect of glucose-6-phosphate in a purified brain hexokinase preparation, and extended it to include fructose, 2-deoxy-glucose, mannose and glucosamine as substrates. Similar inhibitory effects were described later by Crane and Sols (1953) using heart, kidney, intestinal mucosa, stomach and liver homogenates and

red cell lysates. It was suggested, on the basis of these findings, that the rate of glucose utilisation by tissues is in part controlled by the hexokinase-phosphofructokinase system. Faster utilisation of glucose-6-phosphate can result in an increase of hexokinase activity, for it is considered that this activity is normally inhibited slightly by glucose-6-phosphate present, so that the production of the ester from other sources results in a decreased glucose utilisation. If this theory is correct it is possible that the depressant action of adrenaline on peripheral glucose utilisation in animals (Somogyi, 1950) is a result of glucose-6-phosphate accumulation, for it has been found that adrenaline injections cause an increase of glucose-6-phosphate concentration in muscle (Cori and Cori, 1931, 1932).

Weil-Malherbe and Bone (1951) found no inhibition of brain hexokinase by hexose diphosphate (presumably fructose-1:6-diphosphate) but Sols and Crane (1953) state that glucose-1:6-diphosphate is inhibitory towards purified brain hexokinase. Rat brain and heart tissues were found, by Paladini (1951), to contain much higher concentrations of glucose-1:6-diphosphate than liver, kidney, intestine, muscle or blood. As it was also found that the glucose diphosphate concentration of muscle was doubled after injection of glucose, adrenaline or insulin, it is possible that glucose diphosphate, acting as a co-enzyme in the reaction

glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate (Leloir, Trucco, Cardini, Paladini, and Caputto, 1948), can also have some control over the rate of hexose phosphorylation by tissue kinases.

Glucosamine produced an inhibitory effect on fructose phosphorylation but not on glucose phosphorylation, in acetone-dried preparations, according to Harpur and Quastel (1949). It was also demonstrated that N-acetyl glucosamine inhibited the phosphorylation of glucose, fructose and glucosamine by this preparation, but the stated competitive nature of this inhibition is not apparent from reported results.

It was stated by Reichstein et al (1950) that 4.1×10^{-4}M L-glyceraldehyde produced almost complete inhibition of a beef brain hexokinase preparation. The preparation used must have been capable of synthesising L-sorbose-1-phosphate in view of the known mechanism of this inhibitory effect, for there is no further evidence of hexokinase inhibition by L-glyceraldehyde. Sols and Crane (1953) reported that L-sorbose-1-phosphate inhibits rat brain hexokinase, in confirmation of the earlier findings of Lardy, Wiebelhaus and Mann (1950) with beef brain hexokinase.

1.2.7.2. Hormonal.

The investigations of the Cori group (Price, Cori, and Colowick, 1945; Price, Slein, Cori, and Colowick, 1946; Colowick, Cori, and Slein, 1947;) on the effects of certain

hormones on muscle hexokinase stimulated a great deal of research in this field as their results offered an explanation of the mechanism of the physiological regulation of carbohydrate metabolism. Many workers (Stadie and Zapp, 1947; Reid, Smith, and Young, 1948; Stadie and Haugaard, 1949; Stadie, Haugaard, and Hills, 1950) have been unable to confirm their findings, however, although several investigators (Christensen, Plimpton, and Ball, 1949; Weil-Malherbe, 1950; Weil-Malherbe and Bone, 1951) have obtained protein fractions from animal and human blood which do not affect hexokinase activity.

These investigations will not be discussed in detail for although the non-specific stabilisation of yeast hexokinase by certain proteins (1.1.5.) has been reported, the effects produced on muscle hexokinase by hormones and certain protein fractions appear to have no parallel with the yeast enzyme.

1.2.8. Conclusions.

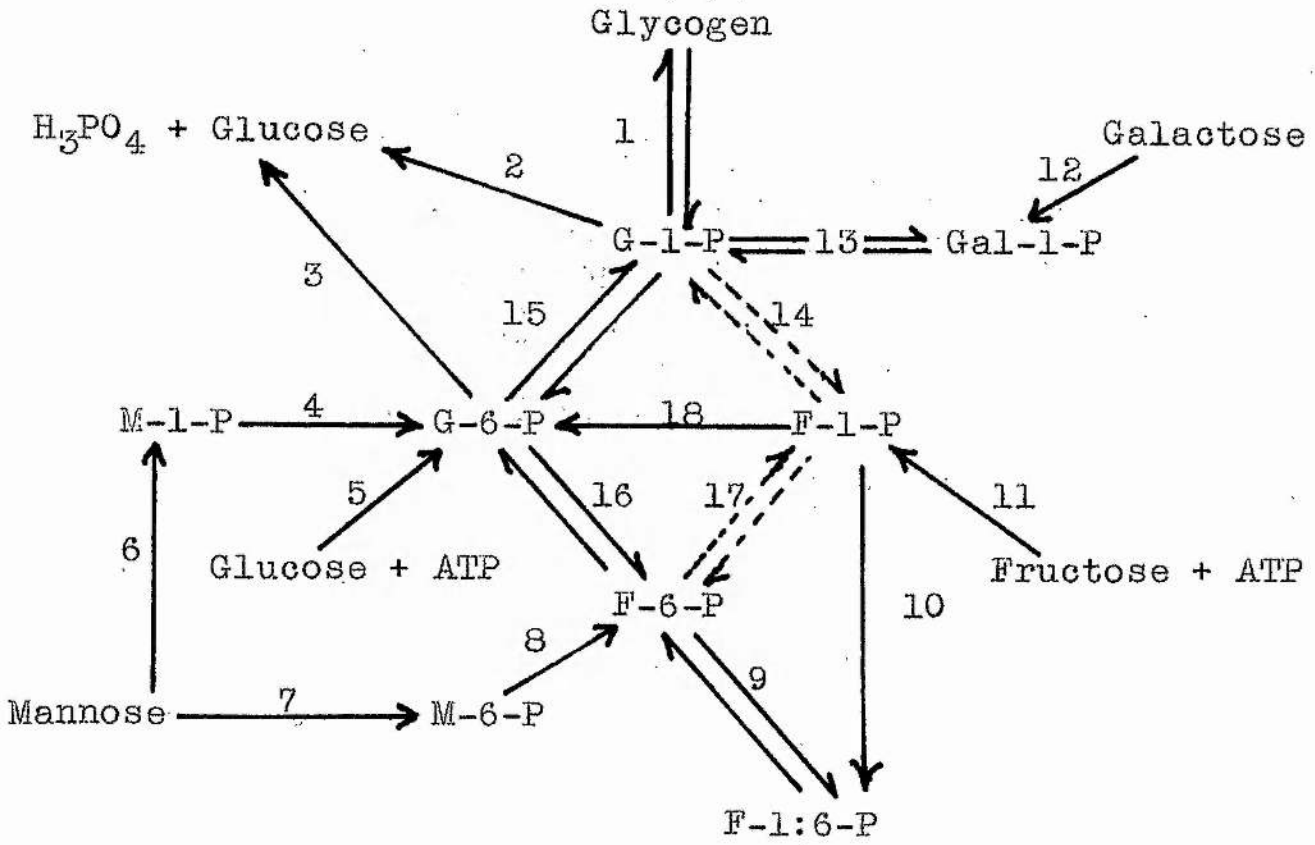
Most studies on mammalian kinases have been carried out using crude tissue extracts or partially purified enzymes, and as the isolation of these preparations varies in detail, it is evident that conflicting results must appear. Much information has been obtained, however, which allows a greater understanding of some of the regulatory mechanisms of tissue glycolysis.

Although no definite conclusions can be made regarding the specificity of various tissue kinases, it is possible to suggest that tissues contain a preponderance of the kinase most suited to the metabolic requirements of that tissue, in addition to other kinases which initiate the metabolism of other less specific substrates.

Brain tissue, for example, according to Long (1950) has a high glucokinase activity, which follows the recognised dependence of this tissue on the level of blood glucose. The inhibition of glucose phosphorylation in brain tissue by glucose-6-phosphate and protein fractions, and its activation by various proteinaceous extracts can also be correlated, to some extent, with known in vivo physiological effects. These findings can be considered only as an indication of possible in vivo mechanisms until more detailed knowledge is obtained.

Liver tissue is shown to have greater fructokinase than glucokinase activity, and the isolation of fructokinase from this tissue has been reported many times. The low glucokinase activity is expected, for glycogenolysis through glucose-1-phosphate probably provides much of the necessary metabolic substrate. The metabolism of fructose, on the other hand, will require a kinase for its primary phosphorylation. Long (1950) has suggested that the formation of glycogen in the liver is through C_3 fragments produced from glucose in the peripheral tissues, a mechanism which would not require a liver glucokinase.

Diagram I.


Glycogen
H_3PO_4 + Glucose
Galactose
1
2
12
G-1-P
13
Gal-1-P
3
15
14
M-1-P
4
G-6-P
18
F-1-P
5
16
17
11
6
Glucose + ATP
Fructose + ATP
F-6-P
10
8
9
Mannose
7
M-6-P
F-1:6-P

The presence of fructokinase in muscle tissue has not yet been explained by any obvious physiological requirements for this enzyme. It has been shown (Slein, 1951) that liver homogenates have a greater fructokinase activity aerobically than anaerobically, and that the utilisation of glucose by rat liver homogenates is the same under aerobic and anaerobic conditions. Should these findings apply to muscle tissue it is possible that fructokinase exerts a glucose-sparing action during muscular activity, or provides extra phosphorylated hexose from which high energy phosphate is eventually produced.

The inter-relationships of the various phosphorylated sugars are shown in diagram I. and the key position of glucose-6-phosphate in carbohydrate metabolism is evident. Normal glycolysis which promotes synthesis of fructose-diphosphate diverts glucose-6-phosphate away from carbohydrate synthesis, but the utilisation of available inorganic phosphate may cause deposition of glycogen, also at the expense of glucose-6-phosphate. When Price, Cori, and Colowick (1945) demonstrated that aerobic phosphorylation was necessary for phosphokinase synthesis of glucose-6-phosphate, and its conversion to glycogen via glucose-1-phosphate, the link between respiration and resynthesis was definitely established. The rate of glycogen synthesis is known to depend directly on blood sugar concentrations, however, for Ostern, Herbert,

and Holmes (1939) have shown that the dephosphorylation of glucose-6-phosphate is inhibited at fairly high glucose concentrations.

Although the formation of glucose-6-phosphate from fructose-1-phosphate has been demonstrated by Cori, et al (1951) it has not been established whether glucose-1-phosphate or fructose-6-phosphate is the primary product in this conversion (diagram I., reactions 14, 17). Slein et al (1950) have shown that fructose-1-phosphate is further phosphorylated by an enzyme in muscle extracts, with the formation of fructose-diphosphate (reaction 10).

There is as yet no definite evidence to show how mannose enters the glycolytic cycle. Leloir (1951) has described a phosphoglucomutase isolated from muscle tissue, which in the presence of catalytic amounts of glucose-1:6-diphosphate transforms mannose-1-phosphate into glucose-6-phosphate. As Slein (1950a) has stated that mannose-6-phosphate is readily converted to fructose-6-phosphate or glucose-6-phosphate by a phosphomannose isomerase, present in rabbit muscle extracts, mannose may enter the glycolytic cycle either through the 1- or 6- phosphate (Reactions, 5, 6, 7, 8).

The co-enzyme for the phosphoglucomutase reaction (reaction 15) was identified as glucose-1:6-diphosphate by Leloir et al (1948), and later this same enzyme was shown

to catalyse the change galactose-1-phosphate $\rightleftharpoons$ glucose-1-phosphate when uridine diphosphate glucose was present as a co-enzyme. (Cardini, Paladini, Caputto, and Leloir, 1950). This finding finally established the method by which galactose is introduced into the glycolytic system.

This brief outline of the routes by which the hexoses and phosphates are introduced into the glycolytic cycle demonstrates the complexity of the system which exists *in vivo*. It is against this background that the demonstrable, *in vitro* inhibition of phosphokinases by various substances, considered to have *in vivo* physiological significance, must be viewed.

1.3. Bacterial, Fungal and Plant Hexokinases.

1.3.1. Introduction.

Little evidence has been presented regarding the hexokinases contained in bacteria, yeasts other than S. cerevisciae, and higher plants. It has been established, however, that the primary phosphorylation of hexoses occurs, in several instances, by the catalytic action of various hexokinases.

1.3.2. Bacterial Hexokinases.

Klein and Doudoroff (1950) isolated a mutant of Pseudomonas putrefaciens which oxidised glucose rapidly, whereas the parent strain was unable to oxidise the sugar. They showed that this phenotypic difference was due to the formation of a glucokinase in the mutant. Their attempts to obtain a fructose mutant were not successful.

A gluconate-adapted strain of Escherichia coli was isolated by Cohen (1950) who reported the presence of a glucokinase in extracts of this organism. The enzyme, which was partially purified, did not catalyse the phosphorylation of mannonate, 2-ketoglutarate, galactonate, idonate or altronate. Cardini (1951) separated a glucokinase from non-adapted Esch. coli which catalysed the phosphorylation of mannose, at a much lower rate.

Fructokinase and galactokinase were also identified in these extracts. Cardini (1951) was able to demonstrate only glucokinase activity in Staphylococcus aureus extracts although the organism is able to metabolise fructose and glucose with equal facility. This author suggested that fructokinase activity was not measurable in these extracts because of the lability of the enzyme.

1.3.3. Fungal Hexokinases.

Grant (1935) found that galactose-adapted yeast cells yielded extracts which were capable of fermenting galactose, but not galactose-6-phosphate. It was reported later by Kosterlitz (1943) that galactose-1-phosphate, which he had shown was formed in the liver of galactose-fed rabbits, was fermented by galactose-adapted yeast. He proposed that the primary phosphorylation of galactose, in yeast and in animals, occurred on C1. This proposal was verified by Trucco, Caputto, Leloir, and Mittlemann (1948) who demonstrated the presence of galactokinase in galactose-adapted brewers' yeast and in the galactose-fermenting yeast, S. fragilis.

A partially purified galactokinase was obtained from extracts of air-dried S. fragilis cells which were treated with bentonite, to remove the bulk of the hexokinase present, and then fractionated with ammonium sulphate.

Using this purified enzyme these authors found that 1.5×10^{-2}M galactose and 2.0×10^{-3}M ATP were required for half saturation. Mn^{++} or Mg^{++} were required for complete activity of the enzyme which had a pH optimum of 6.0.

1.3.4. Plant Hexokinases.

Much of the evidence which has been presented describing the presence of hexokinases in plants is inconclusive.

Griffiths (1949) reported that hexokinase occurred in potato tubers but he measured only ATP uptake in crude potato extracts; this is not direct evidence for hexokinase activity. He also reported that alloxan did not inhibit ATP uptake by these extracts, although it did so in crude extracts of rat muscle. He suggested, therefore, that potato hexokinase had no functional requirement for -SH groups.

The presence of hexokinase in potato tubers was later reported by Kotelnikoona (1951) who also measured the change in 7-minute-labile phosphate in crude extracts. Meeuse, van der Eijk and Latuasan (1942) reported, without giving any details, that hexokinase was present in potato tubers.

Saltman (1953) in a more complete investigation of various plant tissues reported that hexokinase activity

could be demonstrated in wheat germ, mung bean seed, potato tubers, pea seed, Avena seed and spinach leaves. The activity was assayed by measuring the disappearance of free hexose or by the manometric method of Colowick and Kalckar (1943). Wheat germ was found to have the highest hexokinase activity of all the plant tissues tested, and the soluble enzyme from this source was partially purified, by acetic acid precipitation of inert protein and fractionation by ammonium sulphate.

Mg^{++} and Mn^{++} ions activated the enzyme, which was inhibited by Cu^{++}, Zn^{++}, and Hg^{++}ions. Glucose, fructose, mannose and glucosamine were phosphorylated at relative rates of 1.00: 0.62: 0.68: 0.52. Neither galactose, ribose, arabinose nor ribulose was phosphorylated by this preparation. It was also reported that neither iodoacetic acid, iodoacetamide, p-chloromercuribenzoate nor potassium dihydrogen arsenate had any appreciable inhibitory effect on the activity of the enzyme, which indicated that the enzyme was not dependent on -SH groups for its activity.

2. Experiments carried out with Hexokinase Preparations.

2.1. Introduction.

The results obtained in Part I. of this investigation suggested that glucosone exerted its inhibitory effect at some early stage in yeast fermentation. This section of the thesis is a study of the effects of glucosone on ATP utilisation in whole yeast cells, and on hexose phosphorylation by ATP, in the presence of hexokinase. A limited study of the specificity of yeast hexokinase was also carried out.

The actively phosphorylating preparation of yeast cells, used in the preliminary experiments, hereafter called "dry ice yeast", is described in Part III. 3.3. The isolation of the partially purified yeast hexokinase used in the phosphorylation experiments is described in Part III. 3.4.

The adenylic acid system is one of the most important mechanisms concerned with transphosphorylation in alcoholic fermentation. The reactions mediated by this system are catalysed by highly specific enzymes, hexokinase being the first of these in the glycolytic sequence, ATP being the only source of phosphate for this reaction.

A variety of methods can be used to measure the rate, or amount of phosphate transfer from ATP to suitable substrates, by the action of hexokinase. Chemical,

manometric and spectrophotometric procedures have all been employed.

Lohmann (1928) was the first to measure phosphorylation by observing the difference in phosphate ester linkage stability between a phosphate donor and a phosphorylated acceptor. The rate of the hexokinase reaction can be followed by this method, for the pyrophosphate link of ATP is completely hydrolysed by heating for seven minutes in N-HCl, or twelve minutes in N-H_2SO_4, at 100°, whereas the hexose-6-phosphates are not hydrolysed under these conditions. This procedure has been used by several investigators (Bailey and Webb, 1948; Griffiths, 1949: Kotelnikoona, 1951), to measure hexokinase activity but has been criticised by Broh-Kahn and Mirsky (1947) who point out that only ATP disappearance is being measured. The method, therefore, does not necessarily measure only hexokinase activity in impure enzyme systems. Long (1950) has also expressed the view that this method is "certain to give erroneously high values" because of the activities of many other enzymes which contribute to the formation of acid groups.

When ATP donates a phosphate group to a sugar molecule, adenosine diphosphate is formed with an hydroxyl group having a strongly acidic character. This change in acidity, being a measure of utilised ATP, has been determined by three methods. Colowick and Kalckar (1943) measured the change in pH directly,

but no real accuracy can be placed on this method as the buffering capacity of the reaction mixture employed must affect the acidity change. The activity of yeast hexokinase in all stages of purification, was measured by Kunitz and MacDonald (1946) using volumetric acidimetry, but the method does not readily lend itself to rate determination. A modification of this method was introduced by Wajzer (1949) who followed the indicator colour change spectrophotometrically. The method is rather complex, however, for control curves are necessary to correct for spontaneous clouding of the solutions and for changes in acidity outside very narrow limits.

A more elegant method was devised by Colowick and Kalckar (1943), who carried out the hexokinase reaction in 0.03M sodium bicarbonate buffer (pH 7.5) and measured the rate of carbon dioxide output manometrically. This method has been widely used in metabolic studies. It suffers the limitation that only ATP breakdown is being measured and not necessarily the hexokinase reaction.

The simplest, direct determination of hexokinase activity is the measurement of hexose disappearance by any of the normal copper reduction methods, after removal of the reducing hexose phosphates with zinc hydroxide by the method of Somogyi (1945). This is the only simple method which measures hexokinase activity directly, in impure preparations of the enzyme.

Over a period of years, Warburg introduced several spectrophotometric methods for the measurement of transphosphorylation. The methods are all based on the oxidation or reduction of pyridine nucleotides, brought about by a chain of reactions, of which the transphosphorylating reaction is the limiting one. The rate of disappearance of the absorption band of reduced pyridine nucleotides at 340mμ then becomes the measure of the rate of phosphorylation. The rate of glucose phosphorylation can also be measured by adding an excess of "Zwischenferment" (glucose-6-phosphate dehydrogenase) and TPN-cytochrome reductase to the hexokinase system, and following the rate of appearance of reduced cytochrome c (Hogness, 1942). A similar method was used by Slein et al (1950), who measured the rate of reduction of TPN in the presence of excess "Zwischenferment" and phosphohexose isomerase. Another spectrophotometric method introduced by Kalckar and Shafran (1947) measured the amount of ADP formed in phosphorylation reactions. By adding excess myokinase and 5-adenylate deaminase to the hexokinase system, the change in absorption at 265mμ accompanying the conversion of adenosine diphosphate to inosinic acid is a measure of the rate of phosphorylation.

In the present investigation phosphorylation was measured by the manometric method of Colowick and Kalckar (1943). The accuracy of the results was checked where possible by measuring carbohydrate uptake.

2.2. Methods.

2.2.1. Using Dry Ice Yeast.

0.5M potassium fluoride was added to a 5.4% suspension of dry ice yeast in water, to give a final potassium fluoride concentration of 4.8×10^{-4}M. The mixture was shaken for 30 minutes and the pH adjusted to 7.5 by addition of solid sodium hydrogen carbonate. Each Warburg flask contained 1.0ml. of fluoride-treated yeast suspension, 0.45ml. of 0.1M $NaHCO_3$, and 0.6ml. of 0.05M substrate, in the main compartment. The flasks were filled with 5% CO_2/95% N_2 and after equilibration at 30° for 5 minutes, 0.2ml. of 0.04M ATP and 0.1ml. of 0.1M $NaHCO_3$ were added from the side limb.

The carbon dioxide output was measured over the period 0-15 minutes of the experiment.

There was some production of CO_2 in the absence of any substrate, or in the presence of 0.01M galactose. The experimental results reported here in tabular form are all corrected for this blank phosphorylation.

2.2.2. Using Partially Purified Hexokinase Preparations.

The rate of phosphorylation was determined manometrically, again by the method of Colowick and Kalckar (1943), and also in a few experiments, by measuring substrate uptake.

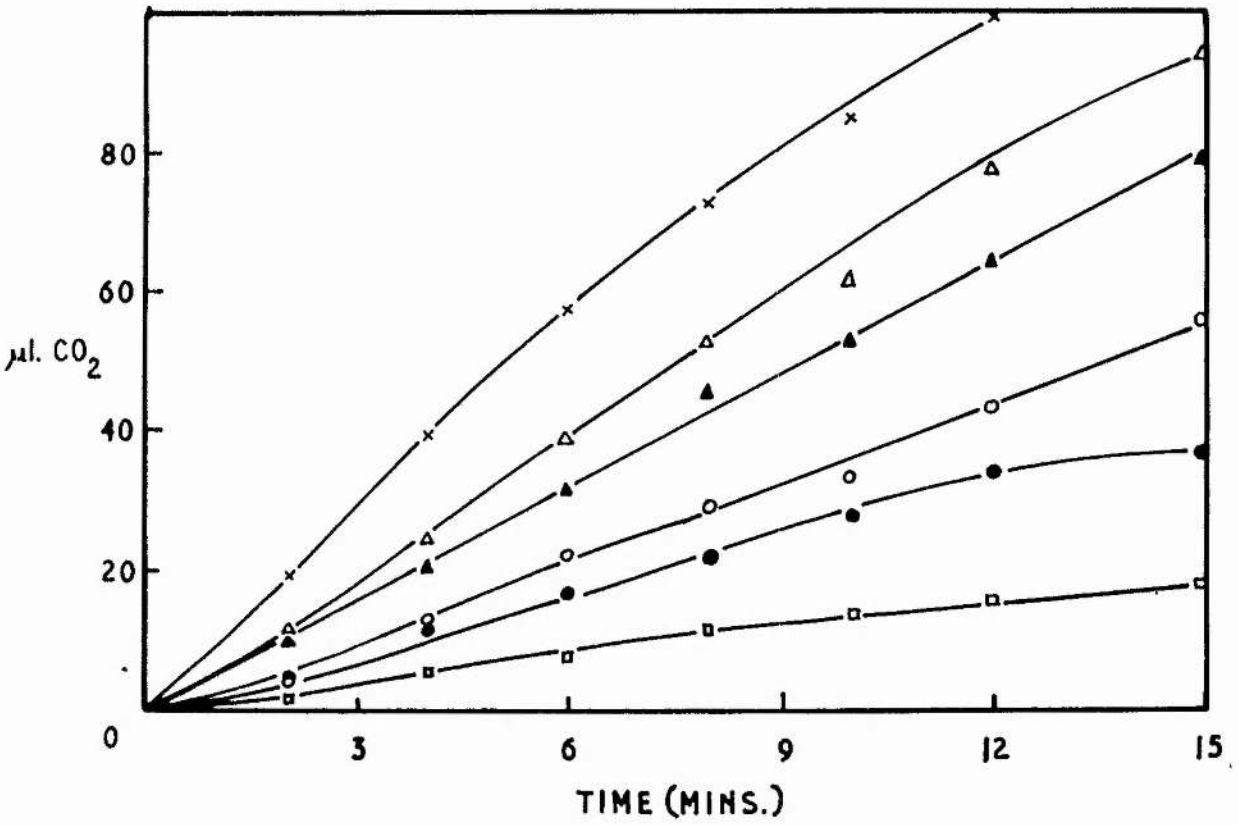
The total fluid volume was 2.0ml. in the manometric experiments, which were carried out at 30°. The main compartment of the Warburg flasks contained 0.2ml. of 0.1M $MgCl_2$, 0.2ml. of 0.05M substrate, 0.4ml. of 0.1M $NaHCO_3$, 0.2ml. of 0.5M KF, and 0.65ml. of neutralised enzyme solution, added in that order. After filling the flasks with nitrogen-CO_2 mixture, and equilibration at 30° for 5 minutes, 0.25ml. of 0.04M ATP and 0.1ml. of 0.1M $NaHCO_3$ were added from the side limb at zero time.

The carbon dioxide output was measured for the first 8-10 minutes of the experiment.

Hexose uptake was measured by the method of Nelson (1944) using the modified reagents described by Somogyi (1952).

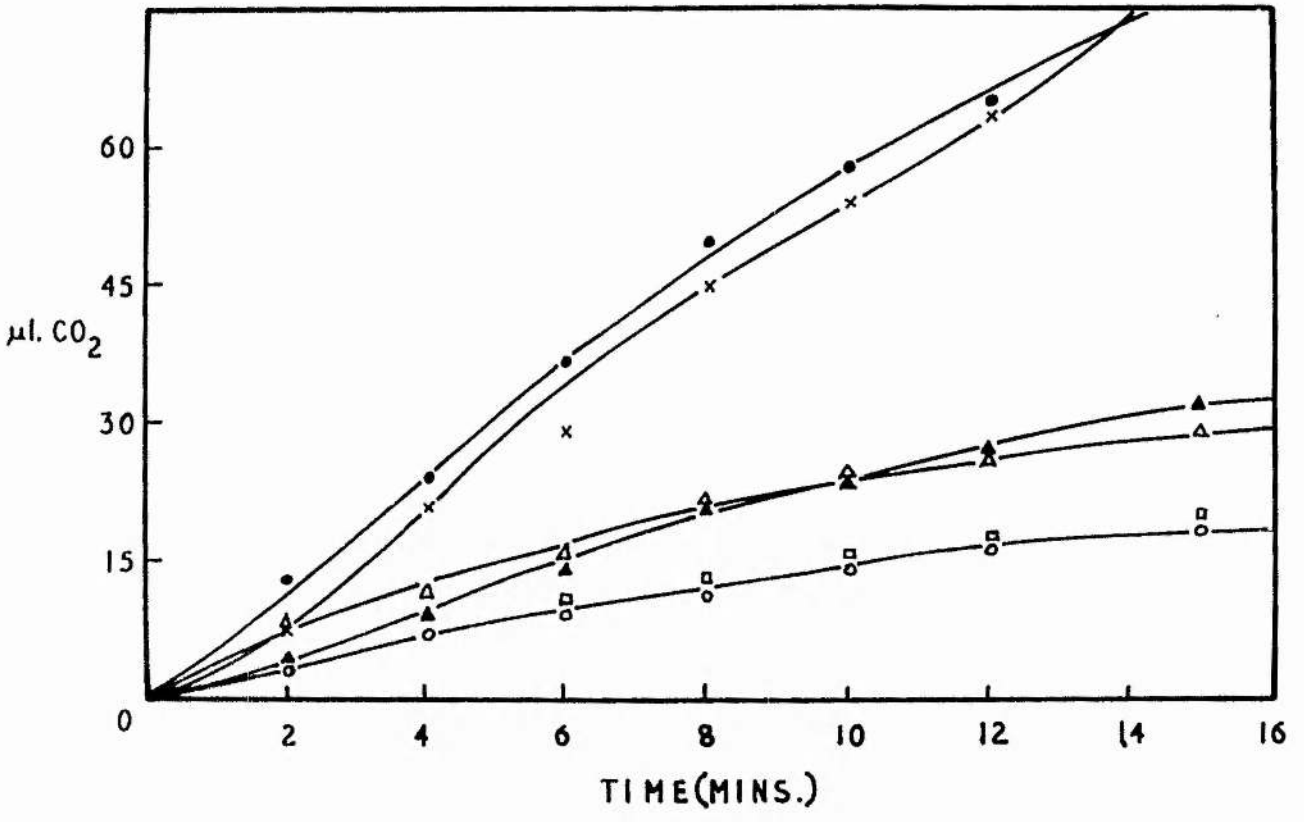
The enzyme preparation used for the glucosone experiments was that described in Part III. 3.4.3. A more highly purified enzyme was used in the specificity experiments, being fraction 6 described in Part III. 3.4.4.

Fig.XVII. Effect of D-glucosone on ATP breakdown.



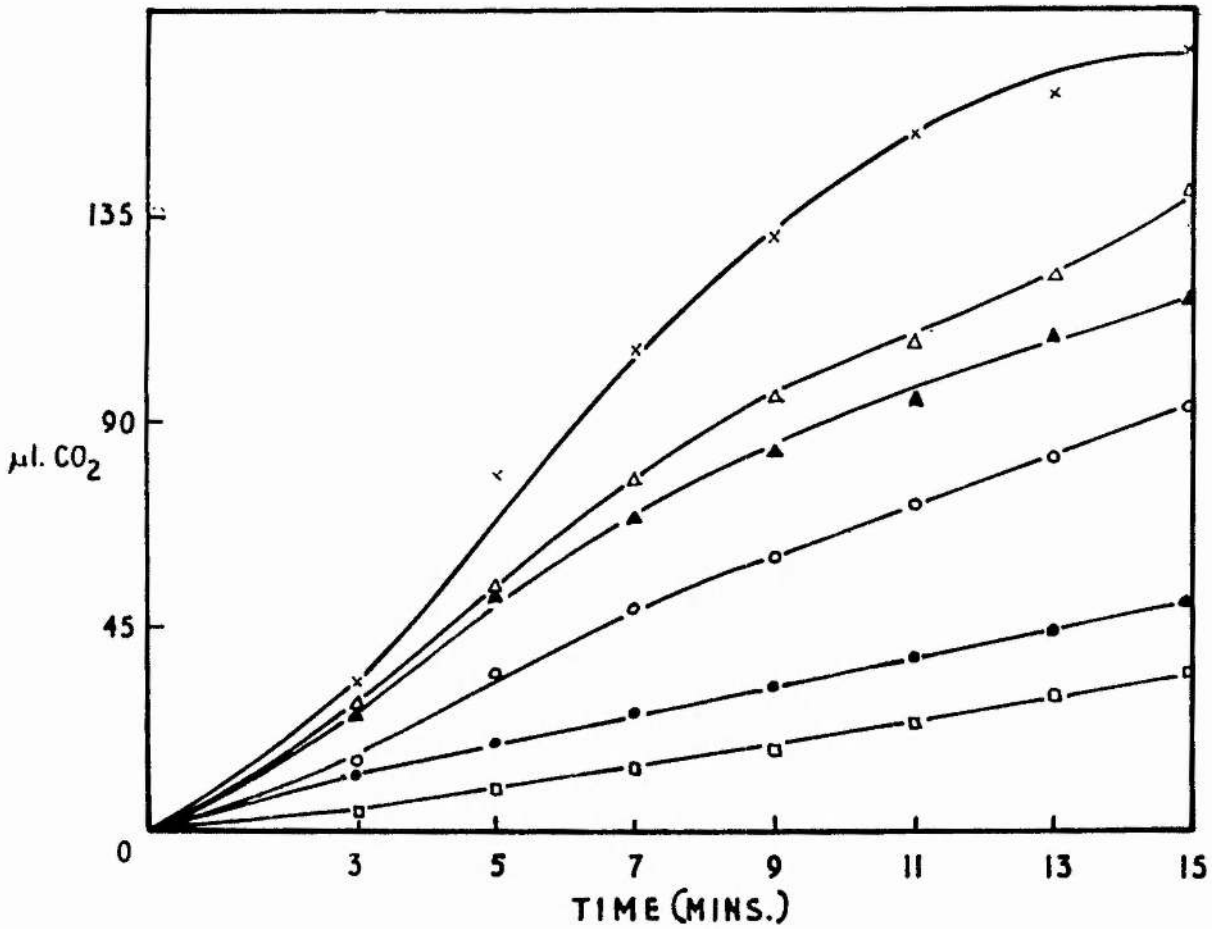
0.003M ATP, 0.01M D-glucose. ×—× , no glucosone; Δ—Δ , 0.008M D-glucosone; ▲—▲ , 0.02M D-glucosone; o—o , 0.03M D-glucosone; ●—● , 0.03M D-glucosone, no glucose; □—□ , no glucose, no glucosone.

Fig.XVIII. Effects of D- and L-glucosone on ATP breakdown.



ATP 0.003M, D-glucose 0.01M. ×—× , no glucosone; ●—● , D-glucose and 0.067M L-glucosone; Δ—Δ , 0.067M D-glucosone; ▲—▲ , D-glucose and 0.067M D-glucosone; ○—○ , 0.067M L-glucosone; □—□ , no D-glucose, no glucosone.

Fig.XIX. Effect of D-glucosone on ATP breakdown, at high concentration of ATP.



0.009M ATP, 0.01M D-glucose. ×—× , no glucosone; △—△ , 0.008M D-glucosone; ▲—▲ , 0.02M D-glucosone; ○—○, 0.03M D-glucosone; ●—● , 0.03M D-glucosone, no glucose; □—□ , no glucose, no glucosone.

2.3. Results.

2.3.1. Using Dry Ice Yeast.

The rate of ATP breakdown by dry ice yeast in the presence of potassium fluoride is shown in Fig.XVII. In the presence of glucose, ATP was utilised readily but addition of D-glucosone inhibited nucleotide breakdown. D-Glucosone exerted a much greater inhibitory effect on this system than on fermentation, but did not appear to be phosphorylated under these conditions.

The inhibition was again specific for the D-isomer, for L-glucosone produced no effect on ATP uptake using glucose as the phosphate acceptor (Fig.XVIII.). The concentration of ATP present was not a limiting factor in these experiments (Fig.XIX.).

The inhibitory effects of D-glucosone on ATP uptake using glucose, fructose, mannose and glucosamine as substrates are shown in Table VI.

TABLE VI.

Substrate (0.01M)	D-Glucosone (M)	µl CO_2 released in 15 min.
Glucose	-	57
	0.02	19
Fructose	-	67
	0.02	14
Mannose	-	55
	0.02	22
Glucosamine	-	25
	0.02	21

(Data from Appendix, Tables 31 and 32)

It was noticed later that there was always some carbon dioxide output at the higher concentrations of the D-glucosone. The rate of ATP breakdown was similar for 0.067M D-glucosone and 0.01M D-glucose plus 0.067M D-glucosone (Fig.XVIII.) which suggested that D-glucosone phosphorylation occurred. Further experiment showed that a significant amount of ATP was utilised in the presence of concentrations of D-glucosone above 0.04M. The rate of ATP breakdown was

much lower than that which occurred in the presence of similar concentrations of glucose (Table VII.).

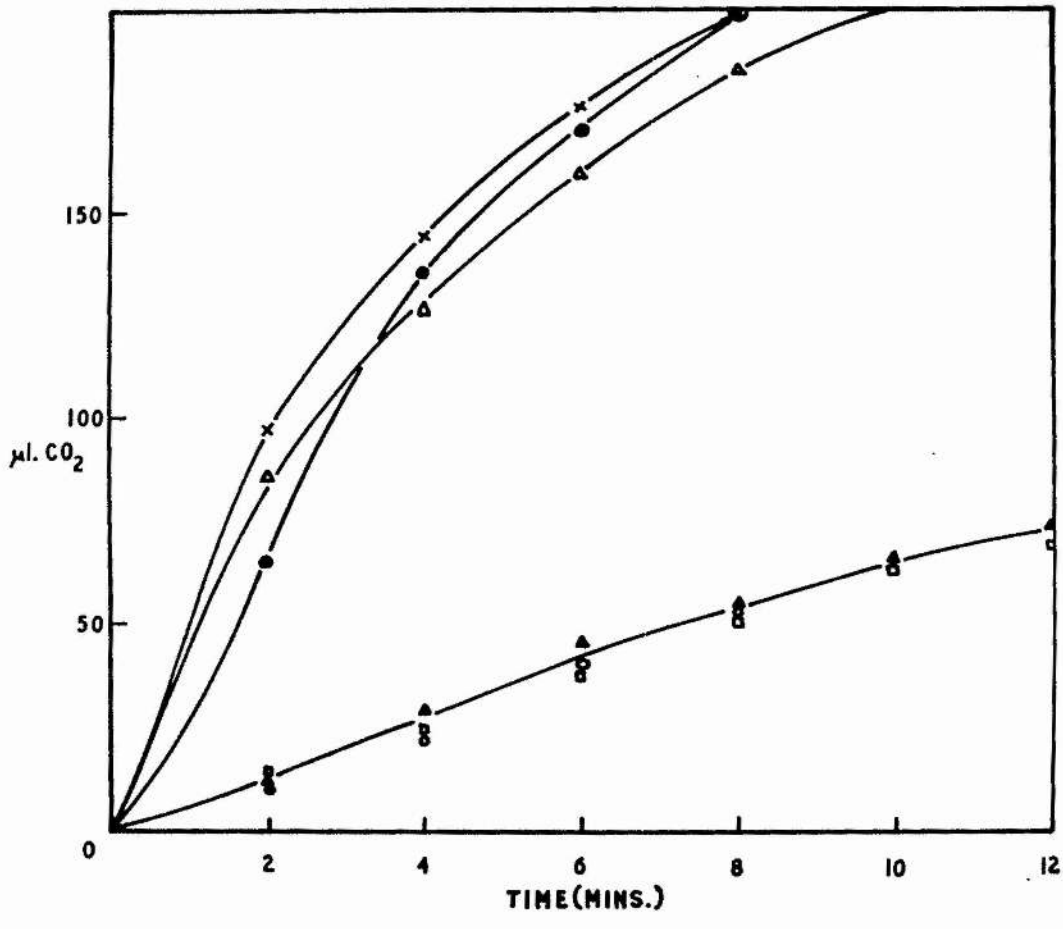
TABLE VII.

Concentration (M)	D-Glucose	D-Glucosone
	μl CO_2 released in 15 min.	
0.01	78	9
0.02	90	13
0.03	93	17
0.04	87	24
0.05	84	30
0.067	89	11
0.075	85	23

(Data from Table 36 of the Appendix)

The significance of these findings and the mechanism of the glucosone inhibition of ATP breakdown is discussed in Section 2.4.1.

Fig.XX. Phosphorylation of D-glucosone by partially purified hexokinase.



×—×, 0.005M D-glucose; Δ—Δ , 0.005M D-glucosone; ●—●, 0.005M D-glucose and 0.005M D-glucosone; ▲—▲ , 0.005M L-glucosone; ○—○ , 0.005M D-galactose; □—□ , 0.005M no substrate.

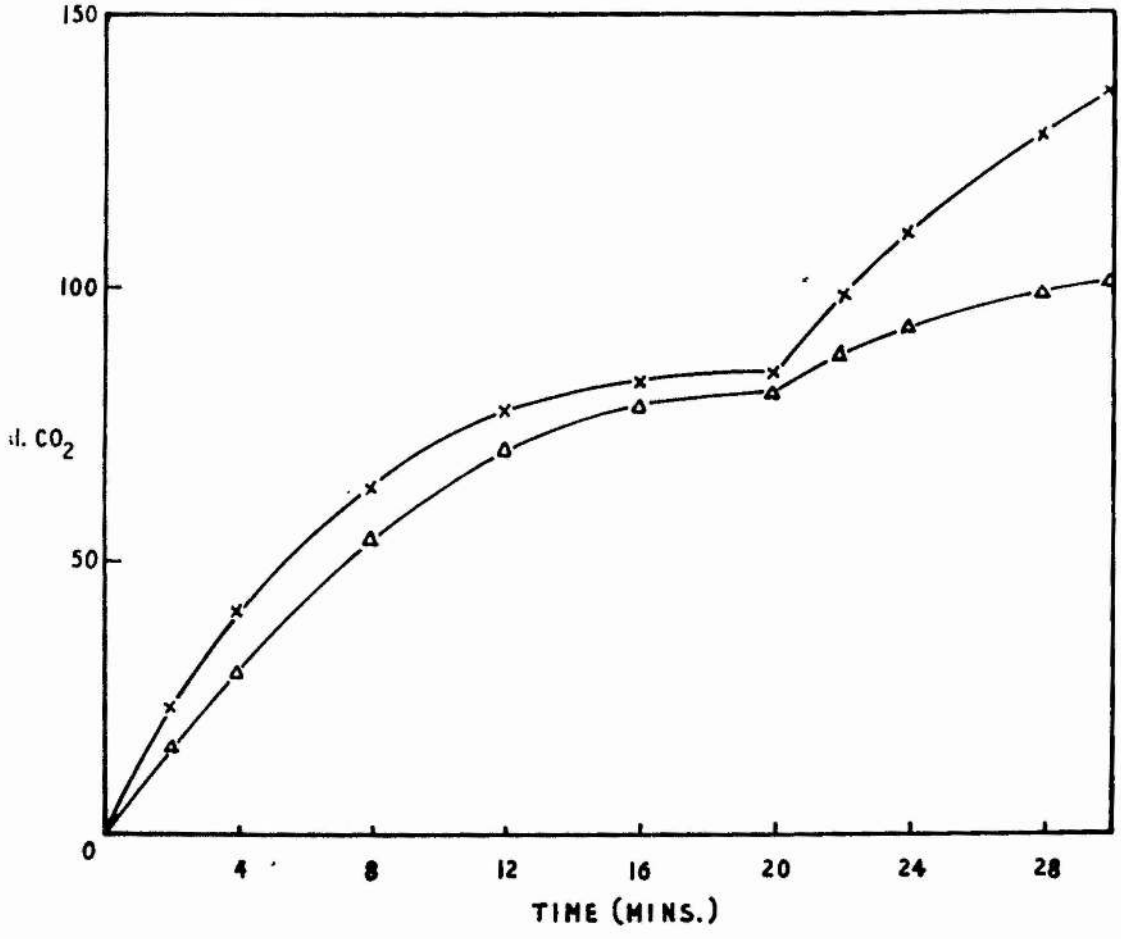
2.3.2. Using Partially Purified Hexokinase.

2.3.2.1. Experiments with Glucosone.

D-glucosone was phosphorylated at only a slightly lower rate than glucose using the preparation described in Part III. section 3.4.5. (Fig.XX.). Neither D-galactose nor L-glucosone was phosphorylated when tested under identical conditions. A mixture of equimolar quantities of D-glucose and D-glucosone was phosphorylated at the same rate as pure D-glucose (Fig.XX.). Any competitive effect which might occur between the two sugars for hexokinase was, therefore, not obvious under these conditions.

The rate of phosphorylation was also determined by measuring the rate of disappearance of the sugars at varyihg time intervals. Results obtained by this method are compared with thoseobtained by the manometric method in Table VIII. The manometric results tabulated are those shown in Fig.XX.

Fig.XXI. Effect of glucosone phosphate on hexokinase activity.



Both curves blank subtracted. ×—× , D-glucose 0.0025M, 0.0025M D-glucosone and ATP added at 20 min. Δ—Δ , D-glucosone 0.0025M, 0.0025M D-glucose and ATP added at 20 min.

TABLE VIII.

Time min.	Glucose		Glucosone	
	CO_2 output (μM)	Hexose uptake (μM)	CO_2 output (μM)	Sugar uptake (μM)
2	3.41	3.11	2.95	2.92
4	4.92	4.23	4.12	3.95
8	5.90	5.12	5.35	4.94
12	6.00	5.65	5.75	5.39

As glucosone is phosphorylated by hexokinase, the inhibitory effects of the osone on fermentation may be caused either by a non-removal of glucosone phosphate from the enzyme molecule, or by the inhibition of some other enzyme by the enzyme phosphate. The inhibitory effect of the glucosone phosphate on hexokinase activity is indirectly demonstrated in Fig.XXI.

Low concentrations of glucosone and of glucose were phosphorylated in the presence of excess ATP for 20 minutes. Glucosone plus ATP was then added to the flask containing phosphorylated glucose, and glucose plus ATP to the other flask containing phosphorylated glucosone. The added glucosone was phosphorylated at a higher rate than the added glucose, which indicated that the primary

PLATE 1.

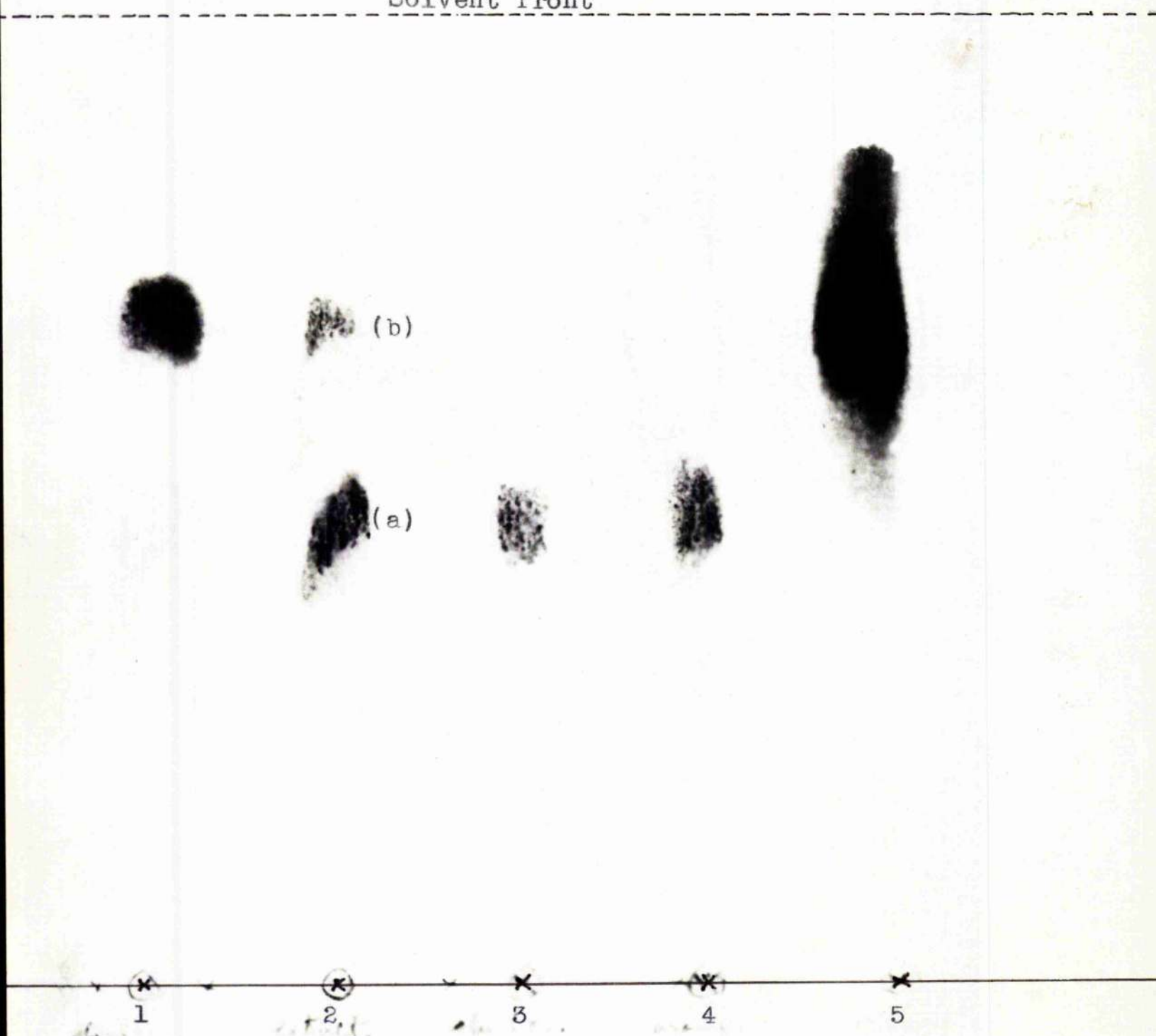


PLATE 1.

1. Glucose.
2. Glucose-6-phosphate (a) formed from glucose (b) after treatment with hexokinase and ATP.
3. Glucose-6-phosphate.
4. Glucosone-phosphate formed from glucosone, after treatment with hexokinase and ATP. The non-appearance of the glucosone is discussed on p.191.
5. Glucosone.

The chromatogram was run on Whatman No.2 paper, in ethyl acetate-amylalcohol-formamide (1:2:3), for five hours at 40°.

Spray?

PLATE 2.

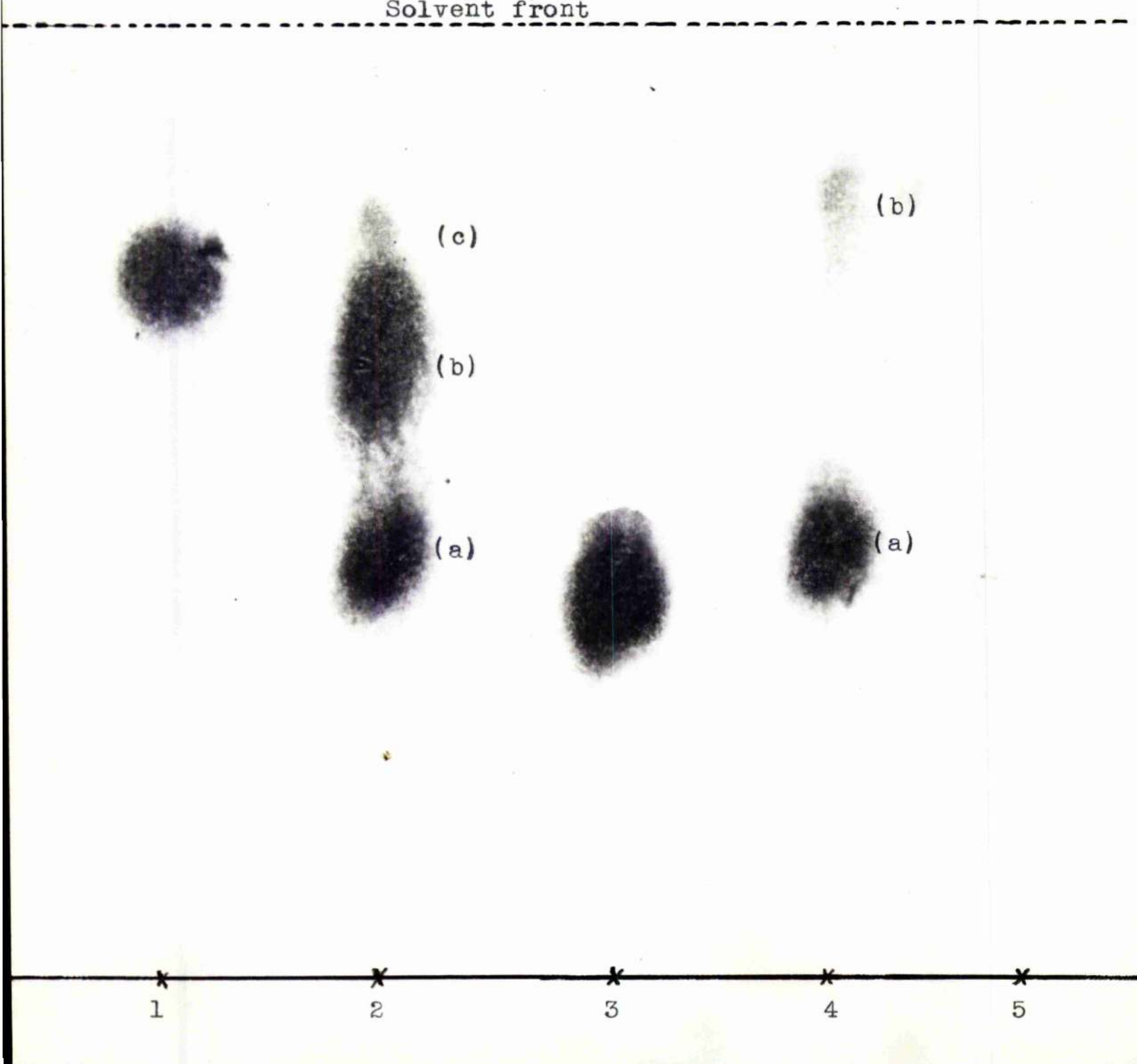


PLATE 2.

1. Glucose treated with hexokinase without ATP.
2. Glucose-6-phosphate (a), glucose (b), and glucosone (c).
3. Fructose-1-phosphate.
4. Glucosone phosphate (a) formed from glucosone (b) treated with hexokinase and ATP.
5. Glucosone which did not appear, treated with hexokinase without ATP.

Chromatographic procedure as for Plate 1.

PLATE 3.

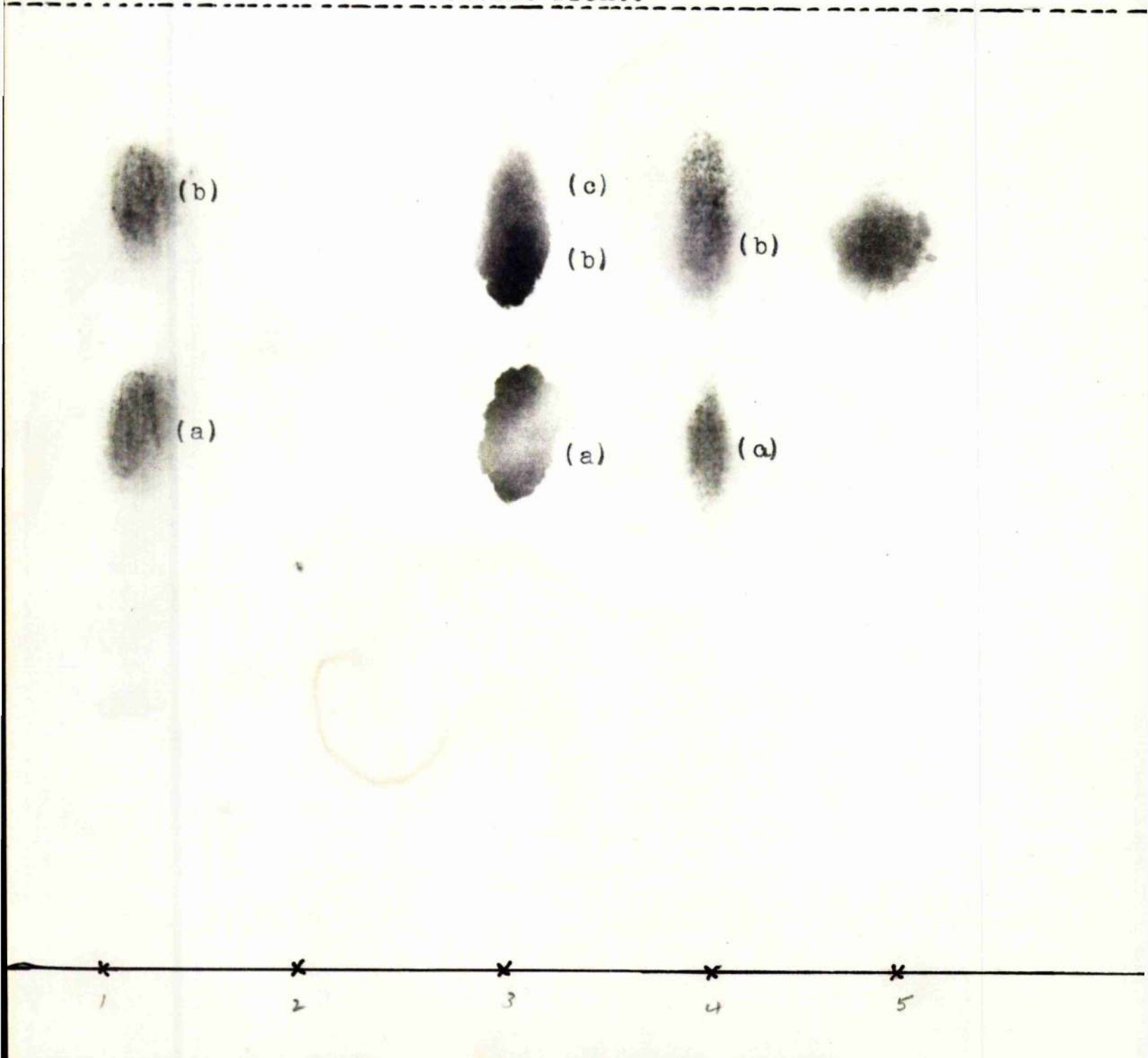


PLATE 3.

1. Glucose-6-phosphate (a) formed from glucose (b) treated with hexokinase and ATP.
2. Hexokinase plus ATP, no glucose.
3. Glucose-6-phosphate (a), glucose (b), glucosone (c).
4. Glucosone phosphate (a) formed from glucosone (b) treated with hexokinase and ATP. (b) + (c)
5. Glucose treated with hexokinase without ATP.

Chromatographic procedure as for Plate 1.

phosphorylation of glucosone produced some inhibitory effect on the hexokinase. A direct proof of the inhibitory effects of glucosone phosphate on hexokinase must await the synthesis of the compound by chemical methods. (see Part III. 1.3.3.).

The products of phosphorylation were examined chromatographically with the results shown on plates 1, 2, and 3.

The chromatograms were all developed in an ethyl acetate/amylalcohol/formamide solvent, and reducing substances were detected using triphenyltetrazolium chloride. The chromatographic procedures employed are described in Part III. section 5.

Plate 1 shows the formation of reducing substances from both glucose and glucosone which had similar R_f values to that of glucose-6-phosphate. Glucosone could not be detected on some chromatograms after being phosphorylated under the test conditions, but it was later discovered that the osone did not disappear provided the reaction mixture was acidified after only 10-15 minutes at 30°.

Plates 2 and 3 are control chromatograms showing that no reducing compounds were present in the reaction mixture prior to the addition of glucose or glucosone; and that no substance corresponding to glucose-6-phosphate was formed in the absence of added ATP.

This solvent mixture does not separate fructose-1-phosphate from glucose-6-phosphate (Plate 7, Part III. section 5.

These phosphates were separable to some extent using the solvents, formic acid/acetone (Burrows, Grylls, and Harrison, 1953) or ethyl acetate/pyridine/water (Hanes and Isherwood, 1949). The R_f or R_p values obtained for various sugar phosphates using these solvents and phosphate-detecting sprays are shown in Table IX., as the chromatograms obtained could not be reproduced photographically. The R_p values reported under Hanes and Isherwood (1949) were calculated by the present author from the results reported by them. The R_p is the $\frac{\text{distance travelled by the organic phosphate}}{\text{distance travelled by O-phosphate.}}$

The new reducing substance observed after treatment of glucosone with hexokinase and ATP, appeared as a diffused spot with an R_f value similar to that of glucose-6-phosphate. Although fructose-1-phosphate was not separated appreciably from glucose-6-phosphate with the solvents used, the R_f value was different from that of the glucosone phosphate. Spots which had similar R_f values to those of ADP and ATP appeared on the chromatograms of the glucose and glucosone extracts.

TABLE IX. R_f or R_p values for some phosphate esters in different solvent mixtures.

	Ethyl acetate-pyridine-water		Ethyl acetate-pyridine-water		acetone-35% formic acid	
	Hanes and Isherwood (1949)	present author	Mortimer (1952)	present author	Burrows et al (1953)	present author.
	R_p		R_f		R_f	
Fructose-1-phosphate	-	.53	-	.47	-	.32
glucose-1-phosphate	.48	.46	.44	.37	.30	.28
fructose-6-phosphate	.57	-	.54	-	.40	-
glucose-6-phosphate	.46	.44	.50	.44	-	.36
fructose-1:6-diphosphate	.19	.15	.13	.10	.42	.40
ADP	-	-	-	-	.1a	-
ATP	.08	.05	-	.03	.04	.02
"glucose extract"	-	.44 .04-.08		.43 0-.07 0-.02		.35-.36 .10 0-.03
"glucosone extract"		.48-.50 .03-.08		.43-.45 .06 0-.02		.34-.36 .10 0-.03

"glucose extract": solution obtained after removal of protein and cations from a phosphorylated reaction mixture which contained glucose.

"glucosone extract": solution obtained after removal of protein and cations from a phosphorylated reaction mixture which contained glucosone.

2.3.2.2. Experiments with Various Substrates and Substrate Analogues.

Within the past few years the phosphorylation of three glucose analogues by either partially purified or crystalline hexokinase, has been reported. Brown (1951), and Grant and Long (1952) reported the phosphorylation of glucosamine, Cramer and Woodward (1952) the phosphorylation of 2-deoxy glucose and Johnstone and Mitchell (1952) the phosphorylation of glucosone.

It seemed desirable that a systematic investigation into the structural relationships of hexokinase substrates should be made. The effects of certain hexose phosphates and inhibitors on hexokinase activity were also investigated, as conflicting results regarding the effects of these substances have been reported (Part II., section 1.1.6.).

The rates of phosphorylation of the known hexokinase substrates were determined and are shown in Table X.

PLATE 4.

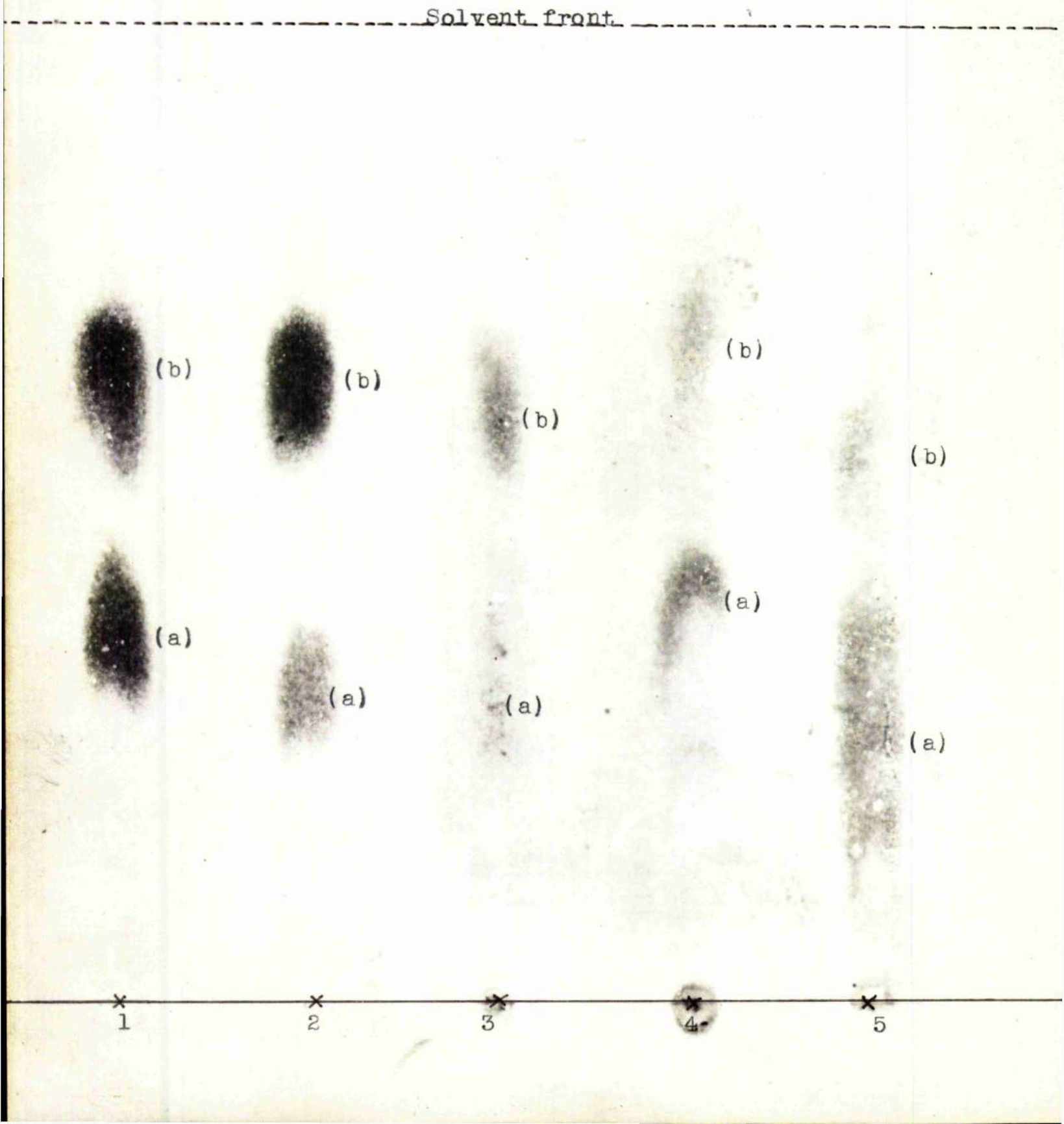


PLATE 4.

1. Glucose-6-phosphate (a) formed from glucose (b) treated with hexokinase and ATP.
2. Fructose phosphate (a) formed from fructose (b) treated with hexokinase and ATP.
3. Glucosone phosphate (a) formed from glucosone (b) treated with hexokinase and ATP.
4. 2-deoxy-glucose phosphate (a) formed from 2-deoxy-glucose (b) treated with hexokinase and ATP.
5. Glucosamine phosphate (a) formed from glucosamine (b) treated with hexokinase and ATP.

Chromatographic procedure as for Plate 1.

TABLE X.

Substrate (0.005M)	Output per 10 min. (μl CO_2)	Rate of phosphorylation (glucose = 1.0)
2-deoxy glucose	172	1.08
Glucose	160	1.00
Fructose	158	1.00
Glucosone	140	0.87
Mannose	132	0.82
Glucosamine	120	0.75

A chromatogram of the products from this experiment is reproduced on Plate 4. The chromatographic procedure was identical with that used previously.

Each extract was resolved into two components by this technique. It was considered that the appearance of the slower-running components was indicative of the formation of phosphorylated derivatives, as the R_f values of these slow-running components were similar to those of the hexose phosphates used previously as control substances. No definite proof of phosphorylation was obtained as the respective sugar phosphates were not all available.

Cramer and Woodward (1952) reported that 2-deoxy glucose was phosphorylated at a rate similar to that of

glucose but in the present experiments the rate of phosphorylation of this sugar was always slightly higher than that of glucose. The phosphorylation rates of glucose and fructose were found to be very similar, although Berger et al (1946) reported that the relative rates were 1. and 1.4. Kunitz and MacDonald, (1946) on the other hand, reported that glucose was phosphorylated at the same rate as fructose. The relative rates of phosphorylation of glucose and mannose were 1. and 0.3 according to Berger et al (1946) and 1. and 0.5, according to Kunitz and MacDonald (1946. More recently Slein et al (1950) have reported that the ratio of mannose to glucose phosphorylation is 0.75 at complete saturation of the enzyme. This figure was obtained after measuring the rate of phosphorylation by a spectrophotometric method. It was suggested that the lower ratio obtained earlier (Berger et al, 1946) using a manometric method, might be due to some impurity present in the mannose which exerted an inhibitory effect on the enzyme. The turnover rate of glucosamine by crystalline yeast hexokinase was similar to that of glucose, according to Brown (1951), and Grant and Long (1952) reported that the amino sugar was phosphorylated at 70 per cent. of the rate of glucose. The findings presented here are in good agreement with the latter report.

The compounds listed in Table XI. were not phosphorylated under the test conditions.

TABLE XI..

D-arabinose	N-acetyl D-glucosamine	1:5 anhydro D-glucitol
D-galactose	α methyl D-glucoside	D-glucose-1-phosphate
L-sorbose	β methyl D-glucoside	D-glucose-6-phosphate
D-galactosone	1:5 anhydro D-mannitol	D-fructose-1-phosphate
D-galactosamine		

These compounds produced no inhibitory effects on glucose phosphorylation at concentrations twice that of the substrate.

2.4. Discussion.

2.4.1. The Phosphorylation of D-Glucosone.

The experiments carried out with dry ice yeast demonstrated that ATP uptake was inhibited by glucosone. The fermentation of normal substrates by this yeast preparation was prevented by the addition of fluoride which probably exerted its main effect on enolase. ATP could, therefore, have been utilised at two metabolic levels in the phosphorylation experiments, namely, for the primary phosphorylation of the substrate and also for the phosphorylation of fructose-6-phosphate. A synthesis of ATP might occur under these experimental conditions provided sufficient pyruvate or acetaldehyde was present to enable the reduced DPN to be reoxidised. It is doubtful whether pyruvate could be present in the yeast preparation for Krebs et al (1952) have shown that such preparations have high carboxylase activity, a finding confirmed by the present author. In the absence of pyruvate it is unlikely that acetaldehyde would be present. Thus, when available DPN is all reduced by the formation of diphosphoglyceric acid from phosphorglyceraldehyde, no further synthesis of ATP can occur. It would appear from the existing evidence, therefore, that an uptake of 2 molecules of high energy phosphate was measured under the conditions of the dry ice yeast experiments. The activity of ATPase and other phosphatases in the preparation is shown in the control experiments.

It was shown in later phosphorylation experiments using partially purified hexokinase that glucosone is phosphorylated at a lower rate than glucose. The rate of ATP uptake using dry ice yeast as the source of phosphorylating enzymes and glucosone as the substrate, would therefore be less than half of that of the normal substrates, if it is assumed that glucosone phosphate does not undergo further phosphorylation. The amount of ATP breakdown in the presence of glucosone was not considered significant in the earlier experiments using dry ice yeast and it was concluded that glucosone was not phosphorylated under these conditions. Further investigations showed, however, that measurable ATP breakdown definitely occurred in the presence of higher concentrations of glucosone.

The inhibitory effect of D-glucosone in these experiments is thus probably a relection of the change in total ATP breakdown produced by the inhibition of hexokinase activity. The formation of a glucosone phosphate which dissociated only very slowly from the hexokinase molecule could reduce the rate of the primary phosphorylation of normal substrates to zero, although not affecting the phosphorylation of formed fructose-6-phosphate. The rate of ATP breakdown in the presence of glucose would, therefore, decrease proportionally with the amount of added glucosone, for a direct competition between glucosone and glucose for the hexokinase molecule probably

occurs under the conditions of these experiments.

Although the phosphorylation of glucosone is directly demonstrable in partially purified hexokinase extracts, the structure of the glucosone phosphate has not yet been ascertained. From a consideration of the structural and chemical similarities between the normal substrates and glucosone it is probable that the osone is phosphorylated at C6.

2.4.2 The Specificity of Hexokinase.

Enzymes are considered to be colloidal proteins which catalyse reactions by first forming a complex with their substrates (Brown, 1902; Michaelis and Menten, 1913). Little is known of the nature of these intermediates but it is generally considered that either specific adsorption complexes or definite molecular compounds are formed, or combination occurs by means of covalent bonds, hydrogen bonds or van der Waal's forces.

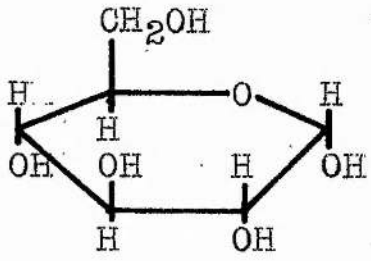
An indication that enzyme-substrate complexes are not formed purely by physical adsorption was obtained when Keilin and Mann (1936) reported that there was a definite interaction between horse radish peroxidase and hydrogen peroxide. Chance (1943) later demonstrated that the peroxidase-hydrogen peroxide complex was relatively stable and he was able to measure the rate of formation and the rate of breakdown of the compound.

It is probable that any phosphorylation of carbohydrate by ATP which is catalysed by hexokinase, requires

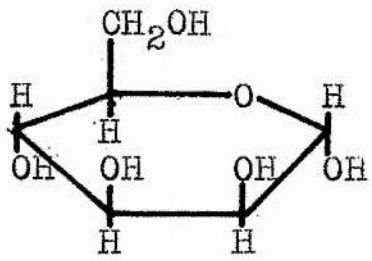
the formation of an intermediate enzyme-substrate complex. It has also been suggested that an ATP-magnesium complex is formed which must be attached to the enzyme molecule before phosphorylation of the substrate can occur. There is little evidence for this hypothesis but definite information has been obtained which defines some structural requirements for the carbohydrate substrates of hexokinase.

The carbohydrates reported to be phosphorylated by ATP in the presence of hexokinase are shown below.

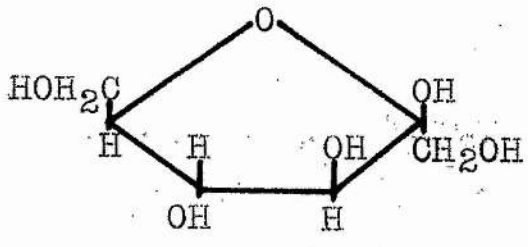
In the ensuing discussion the hydroxyl groups attached to C2, C3, C4, C5 and C6 are described as <u>cis</u> and <u>trans</u> in respect to the reducing hydroxyl in α D-glucose. Taking α D-glucopyranose as an example, the hydroxyl on C2 is <u>cis</u>, that on C3 <u>trans</u> and that on C4 <u>cis</u>.



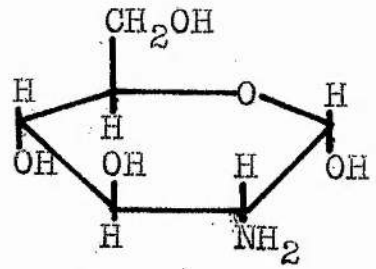
α-D-glucose



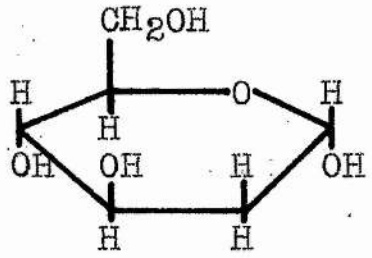
α-D-mannose



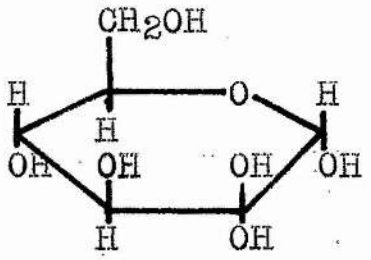
β-D-fructose



α-D-glucosamine



2-deoxy-α-D-glucose



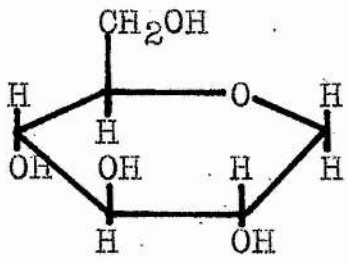
α-D-glucosone hydrate

It can be seen that these carbohydrates have similar configuration of C3, C4, and C5 and have a free reducing group on C1, differing only in the configurations on the substituent at C2.

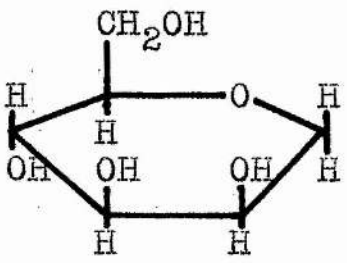
There is no definitive proof of the structure of the osones but from a consideration of the structures of the iso-propylidene derivatives of glucose proposed by Bayne, Collie, and Fewster (1952), it is probable that glucosone itself can exist in the hydrated pyranose form shown above.

Gottschalk (1943) has shown that the α form of glucopyranose is fermented at a slightly higher rate than the β form. He has also reported similar results with α and β mannopyranose (Gottschalk, 1947). These findings indicate that the configuration on C1 has little effect on hexokinase activity.

There is a requirement for a reducing group on C1, however, for 1:5-anhydro-D-glucitol and 1:5-anhydro-D-mannitol, the structures of which are shown below, are not phosphorylated.

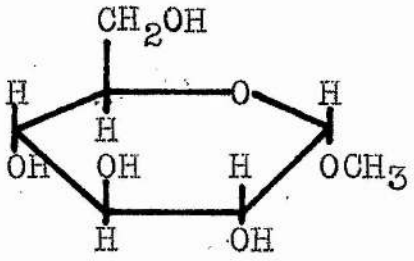


1:5-anhydro-D-glucitol

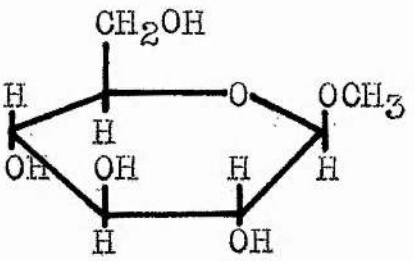


1:5-anhydro-D-mannitol

The formation of a glucoside also prevents phosphorylation by hexokinase, for methyl-α-D-glucoside, and the isomer, methyl-β-D-glucoside are not activated by the enzyme.



methyl-α-D-glucoside

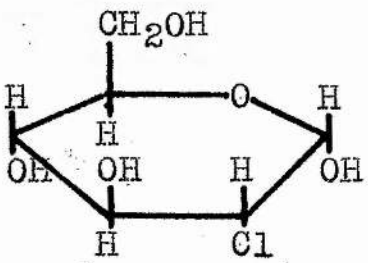


methyl-β-D-glucoside

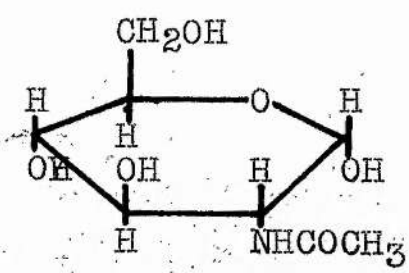
As these compounds are prototypes of all glucosides it is probable that none of the more complex glucosides will be phosphorylated by hexokinase. Kunitz and MacDonald (1946) have reported that neither sucrose, maltose, nor trehalose were phosphorylated by crystalline hexokinase.

Although a configurational change on C2 does not affect the phosphorylation of the hexose, glucose and mannose being both activated, the size and nature of the substituent

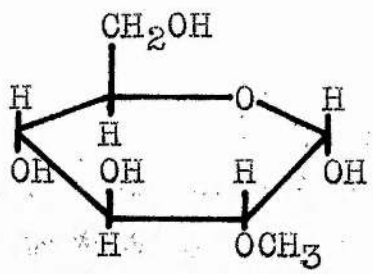
on this carbon atom does affect phosphorylation. Woodward, Cramer, and Hudson (1953) have reported that 2-chloro-2-deoxy-glucose does not inhibit yeast fermentation as effectively as 2-deoxy-glucose. It is possible that the smaller inhibitory effect produced by 2-chloro-2-deoxy-glucose is due to the reduction of the affinity of the parent deoxy sugar for hexokinase, by the introduction of a chlorine atom. The presence of a larger substituent prevents phosphorylation completely, for Brown (1951) has reported that N-acetyl-D-glucosamine was not phosphorylated by crystalline hexokinase, a finding which was confirmed by the present author using partially purified hexokinase. It would be of interest to determine whether 2-O-methyl-D-glucose is phosphorylated in the presence of hexokinase in order to ascertain to what extent the size and the nature of the substituent on C2 are factors determining specificity of the enzyme.



2-chloro-2-deoxy-∝-D-glucose

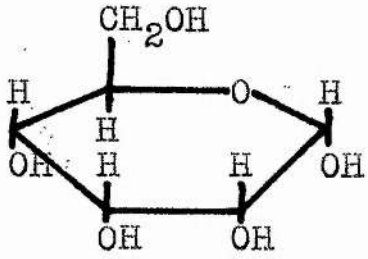


N-acetyl-∝-D-glucosamine

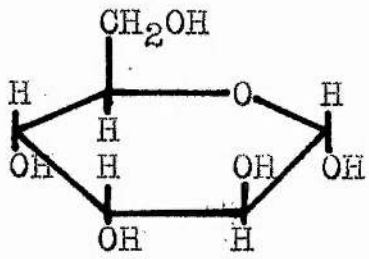


2-O-methyl-∝-D-glucose

All of the sugars which were phosphorylated had the hydroxyl group on C3 in the trans position. No evidence was obtained to show that this configuration was a functional requirement. A test of the ability of hexokinase to catalyse the phosphorylation of D-allose or D-altrose, which are different from D-glucose and D-mannose respectively by the change in configuration at C3, would determine this point.



α-D-allose

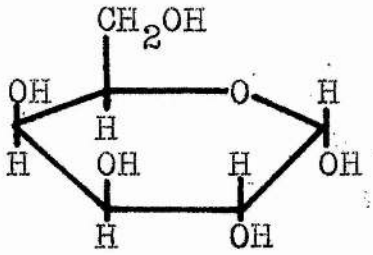


α-D-altrose

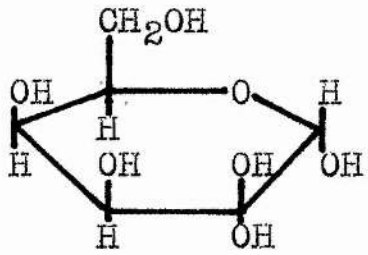
The method used by Richtmeyer and Hudson (1935) for the preparation of altrose does provide some evidence that the cis configuration is required at C3. They hydrolysed neolactose to give altrose and galactose and then removed the galactose by fermentation with brewers' yeast. The non-removal of the altrose would suggest that the sugar is not phosphorylated. This view is not in agreement with that of Gottschalk (1950) who intimated that the configuration at, or the substituent on C3, did not affect the phosphorylative activity of hexokinase. As Wildy (1953) has shown that 3-O-methyl glucose is not phosphorylated by a dry ice yeast preparation, it would

appear that substitution on an hydroxyl group in the trans position on C3 prevents phosphorylation by hexokinase.

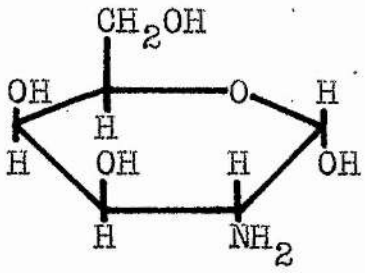
As neither D-galactose, D-galactosone, nor D-galactosamine was phosphorylated, under the test conditions, it is apparent that the hydroxyl group at C4 must have the cis configuration.



α-D-galactose

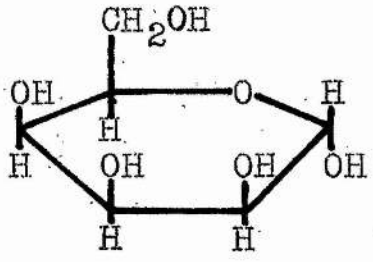


α-D-galactosone hydrate

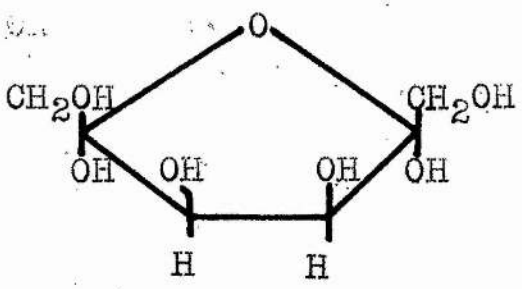


α-D-galactosamine

Further confirmation of the configurational requirement at C4 is given by the fact that neither talose, nor tagatose, which corresponds to mannose and fructose respectively with a change of configuration at C4, are fermented by yeast. (Armstrong and Armstrong, 1934).



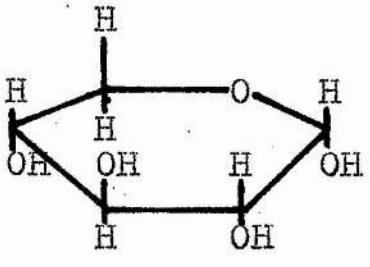
D-talose



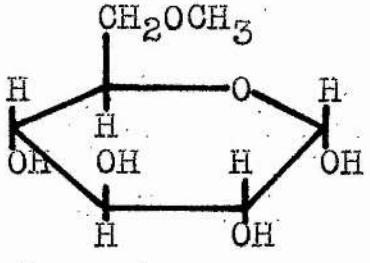
D-tagatose

Substitution on the hydroxyl in the cis position at C4 appears to prevent phosphorylation as maltose and lactose are not affected by hexokinase (Kunitz and MacDonald, 1946).

The hydroxyl group at C6 is the only group which undergoes chemical change in the hexokinase reaction for when it is absent, as in D-xylose (Kunitz and MacDonald, 1946), or substituted as in 6-O-methyl glucose (Wildy, 1953), phosphorylation does not take place.



α-D-xylose

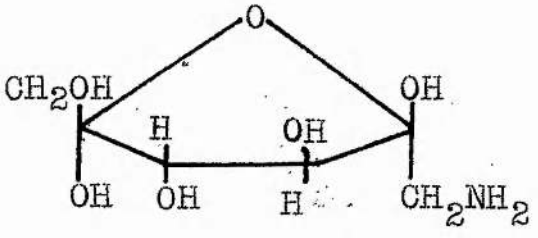


6-O-methyl-D-glucose

Fructose is phosphorylated only in the furanose form (Gottschalk, 1944; Slein, Cori, and Cori, 1950). It is not known whether the α or β isomer, or both, are activated but as the mutarotation of β-D-fructopyranose consists mainly of reaction β-D-fructopyranose ⇌ β-D-fructofuranose (Hudson,

1930), it is assumed that the β isomer is phosphorylated.

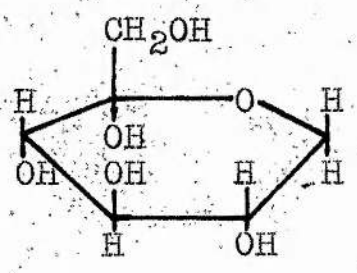
The nature of the specificity of hexokinase is also demonstrated by investigations carried out using fructose analogues. Woodward, Cramer, and Hudson (1953) reported that 1-amino-fructose was more inhibitory towards fructose fermentation than towards glucose fermention. If the mechanism of this inhibition is similar to that of 2-deoxy glucose, 1-amino-fructose must be phosphorylated by hexokinase.



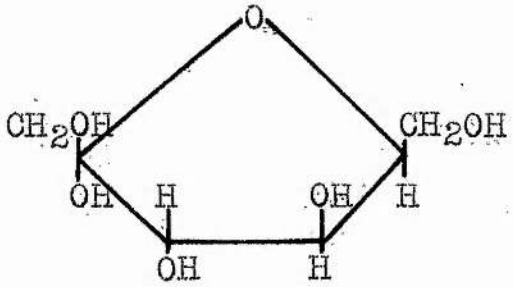
1-amino-α-D-fructose.

As 1-amino-fructose has a free reducing group it can be compared with glucosamine which is also phosphorylated by hexokinase. Similarly 1-O-methyl fructose may be compared with 2-O-methyl glucose. The substitution of the hydroxyls on C3 or C6 of the fructose molecule prevents phosphorylation as in the case of glucose, for Wildy (1953) has reported that neither 3-O-methyl fructose nor 6-O-methyl fructose were phosphorylated by a dry ice yeast preparation. Grieve (1954) has reported that 4-O-methyl fructose, a pyranose derivative of the sugar, is not phosphorylated by partially purified hexokinase.

L-sorbose, which is not phosphorylated, can be considered in the pyranose form as a glucose analogue, or in the furanose form as a fructose analogue. The non-phosphorylation of the pyranose form may be due to the absence of the reducing group at C6 (corresponding to C1 of glucose), or to the presence of a reducing group at C2 (corresponding to C5 of glucose). Similarly the non-phosphorylation of the furanose form may be due to the absence of a reducing group at C2, or to the presence of one at C5.



L-sorbopyranose



L-sorbofuranose

The findings discussed above give an indication that at least four groups are concerned in the attachment of substrates to the hexokinase molecule. Enzyme-substrate attachment by more than one group was first suggested by Armstrong (1904) in the case of glucosidase-glucoside combination. von Euler (1925) later compared the dissociation constant of the invertase-sucrose complex with the product of the affinity constants for invertase-fructose and invertase-glucose, and concluded that invertase made contact with both moieties of

the sucrose molecule. More recently, Pigman (1944), in a review of the glycosidases, considered that combination of enzyme and substrate occurred by attachment at more than one point.

The present investigation appears to confirm the "multiple attachment" theory for the case of hexokinase. However, the assumption made by Gottschalk (1950) that contact between substrate and hexokinase is made through the hydroxyl group on C6 and another hydroxyl group having a cis configuration to the primary alcoholic group, requires modification in the light of the results presentedhere, for the specificity is greater than he suggested.

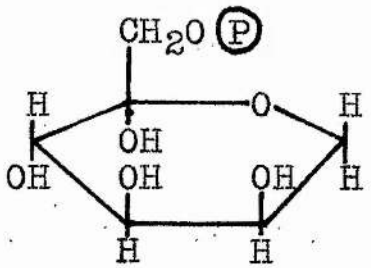
It would appear that if the adsorption area of the enzyme must fit the adsorption area of the substrate, as first visualised by Fischer (1894), then hexokinase requires a specific configuration on each carbon atom, except C1, and C2 of its substrates, before a correct fit can be made.

The attachment of a compound other than a substrate, to the enzyme adsorption area will inhibit, to some extent, the formation of an enzyme-substrate complex. As neither glucose-6-phosphate nor mannose-6-phosphate inhibit glucose phosphorylation, it is probable that these compounds do not attach themselves to the hexokinase molecule. This would imply that when hexose phosphate is formed from hexose on the

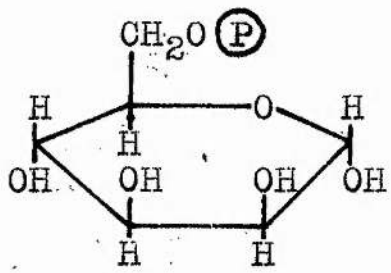
enzyme molecule it dissociates from the enzyme immediately. Although it is generally accepted that enzyme-substrate complexes do break down into enzyme and products very quickly, i.e. the Michaelis constants are determined for enzyme-substrate interaction only, no indication has been obtained of any general mechanism by which the enzyme-product dissociation takes place. The dissociation may occur after the formation of an enzyme-products complex which was formed through an enzyme-substrate complex. Another type of dissociation may be envisaged for certain reactions, however. The change, for example, of glucose into glucose-6-phosphate may be the actual cause of the separation of the sugar phosphate from the active centres of the enzyme. This type of dissociation may occur in enzyme reactions similar to, and including those catalysed by hexokinase, which are virtually irreversible by reason of the large energy change which occurs during the reaction. It is possible that the irreversibility of the reactions is due to the low affinities which the reaction products have for the enzyme.

Although this hypothesis could explain why the normal sugar phosphates do not inhibit hexokinase activity, it would appear to contradict the evidence which has been obtained using glucosone as a substrate for the enzyme, unless the slight changes which may be made in the hexokinase molecule to accommodate the glucosone, prevent the removal of the formed glucosone phosphate.

When Sols and Crane (1953) reported that L-sorbose-1-phosphate inhibited the activity of brain hexokinase, they considered that it exerted its effect in the pyranose form, as in this form it is similar to glucose-6-phosphate, which was also reported to be inhibitory. The absence of any inhibitory effect by fructose-1-phosphate on yeast hexokinase, reported in this thesis, can similarly be correlated with the absence of inhibitory effect by mannose-6-phosphate (Slein, Cori, and Cori, 1950), when the fructose phosphate is considered in the pyranose form.



fructose-1-phosphate



mannose-6-phosphate

Although none of the natural sugar phosphates which have been investigated exert any inhibitory effect on hexokinase, it would be of interest to ascertain whether the 6-phosphates of such compounds as 1:5-anhydro-D-glucitol, 2-O-methyl glucose and 3-O-methyl glucose, inhibit hexokinase activity. An inhibition produced by any of these phosphates would demonstrate an attachment to the enzyme centres, which might not have been apparent from the results of the experiments with the non-phosphorylated compounds.

The inhibition of brain hexokinase by glucose-6-phosphate and L-sorbose-1-phosphate shows that the specificity of that enzyme is rather different from that of the yeast enzyme, but a further investigation, using some of the fructose and glucose analogues discussed above, is required before any real comparison with the yeast enzyme can be made.

It is probable that the hexokinases in yeast, bacteria, plants and animals are similar in some respects, for they must play the same role in all organisms. The variation in the protein structure of different organisms, or different species, or even different organs is, however, a sufficient reason for the observed differences of hexokinase specificity.

PART III.

MATERIALS, ANALYTICAL METHODS

and

CHROMATOGRAPHIC TECHNIQUES

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1. CHEMICAL SYNTHESES.

1.1. Intermediate Compounds.

1.1.1. 1:2-5:6-Di-O-isoPropylidene D-Glucose.

Anhydrous D-glucose (50g.) was shaken with dry acetone (1000ml.) containing concentrated sulphuric acid (30ml.) for 18 hours at room temperature (16.0°). The reaction mixture was neutralised with anhydrous sodium carbonate, with cooling, filtered and evaporated to a thick syrup. Recrystallisation from methanol gave 1:2-5:6-di-O-isopropylidene-D-glucose (58.3g., 81%), m.p.109-110°.

1.1.2. 2:3-4:5-di-O-isoPropylidene-D-fructose.

The products of sucrose hydrolysis were condensed with acetone according to the method of Ohle and Wolter (1930) using concentrated sulphuric acid (4% v/v) as hydrolysing agent and catalyst. After neutralisation with 10N-sodium hydroxide, 2:3-4:5-di-O-isopropylidene-D-fructose was obtained in 52% yield, m.p. 95-96° after recrystallisation from 25% ethanol.

1.1.3. 1:2-O-isoPropylidene-D-Glucose.

(a) Di-O-isopropylidene-D-glucose (190g.) was hydrolysed after the method of Meyer and Reichstein (1946) to give 1:2-O-isopropylidene-D-glucose (128.4g., 79%), m.p.157-159°, after recrystallisation from ethyl acetate.

1.1.3.

(b) D-glucose was acetonated after the method of Mehltretter *et al* (1951).

Concentrated sulphuric acid (80ml.) was added to a stirred suspension of anhydrous D-glucose (100g.) in dry acetone (2000ml.) at 15°, over a period of 10 minutes, care being taken that the temperature did not rise above 25°. After 4 hours stirring at 20-25° the undissolved D-glucose (8g.) was filtered off and washed. The combined filtrate and washings were neutralised with 50% sodium hydroxide (240ml.) with cooling and continuous stirring, so that the temperature was kept below 40°. The sodium sulphate was allowed to settle out overnight, filtered off and acetone washed. The acetone was removed under reduced pressure, water (500ml.) added and the distillation continued. After removal of the acetone, concentrated hydrochloric acid (12ml.) was added to pH 2.0. The mixture was stirred at 40° for 4 hours, cooled and the acid removed by treatment with ion exchange resin I.R. 4B. The resulting liquor was evaporated under reduced pressure to give a faintly yellow syrup from which 1:2-*O*-*iso*propylidene-D-glucose (37.0g., 30%) was obtained, m.p.159-160°, after recrystallisation from ethyl acetate.

1.1.4. 1:2-*O*-*iso*Propylidene-3:5-*O*-Benzylidene-D-Glucose.

1:2-*O*-*iso*propylidene-D-glucose (10g.) mixed with phosphorous pentoxide (8g.) was treated in the cold with

benzaldehyde (24ml.), after the method of Zervas and Sessler (1933). Recrystallisation of the crude product from ethanol gave 1:2-O-isopropylidene-3:5-O-benzylidene-D-glucose (8.0g. 54%), m.p.146.5-147.5°. A mixed m.p. with an authentic specimen of the compound (m.p.148°) gave m.p.146.5-147-5°.

1.1.5. Penta-O-Acetyl-α-D-Glucose.

D-Glucose was acetylated with acetic anhydride, in the presence of anhydrous sodium acetate by the method of Krahl and Cori (1949). Penta-O-acetyl-α-D-glucose was obtained as a white crystalline material m.p.129-131°, in 65% yield.

1.1.6. Tetra-O-Acetyl-α-D-Glucopyranosyl Bromide.

(a) Penta-O-acetyl-α-D-glucose was treated with hydrobromic acid in glacial acetic acid by the method of Freudenberg, Noë, and Knopf (1927), and tetra-O-acetyl-α-D-glucopyranosyl-bromide obtained in 60% yield m.p.80-83°. After recrystallisation from ether m.p.87-88°.

(b) Tetra-O-acetyl-α-D-glucopyranosyl bromide was prepared directly from D-glucose by the method of Barczai-Martos and Körösy (1950) m.p.85-86° in 85% yield. Dry powdered glucose was added to acetic anhydride containing 0.5% perchloric acid over a period of 30 minutes at 30-40°. Powdered red phosphorus was added, the mixture cooled, and bromine added

dropwise over 90 minutes. After the addition of the stoichiometric amount of water the mixture was allowed to remain at room temperature for 3 hours and the product isolated in the usual manner.

Tetra-O-acetyl-α-D-glucopyranosyl bromide was found to be very unstable unless all traces of acid were removed from the product. It is stable for months if kept over calcium chloride, phosphorus pentoxide or sodium hydroxide pellets. According to Isbell and Frush (1950) the addition of 1% of calcium carbonate or barium carbonate to acetobromo-sugars prevents decomposition when the mixture is kept in sealed tubes.

1.1.7. Tetra-O-Acetyl-α-D-Mannopyranosyl Bromide.

D-mannose was acetylated and brominated by the method of Barczai-Martos as described above (1.1.6.(b). The tetra-O-acetyl-α-D-mannopyranosyl-bromide, obtained as a syrup in 84% yield, could not be crystallised.

1.1.8. D-Glucose Phenylosazone.

D-glucose (50g.) added to freshly distilled p-toluidine (35ml.) in 0.5N-acetic acid (100ml.) was heated at 100° until solution was complete. A hot solution of freshly distilled phenylhydrazine (96ml.) in 2N-acetic acid (700ml.) was added with stirring, and the phenylosazone separated out in 5 minutes. The mixture was heated for a further 30 minutes, cooled and filtered. The product was washed with 2N-acetic

acid (twice), water (twice) and ethanol/ether, and dried in a desiccator, in the dark. D-glucose phenylosazone (33.5g., 70%), m.p.206° (decomp.) was obtained as yellow crystals, which on recrystallisation from ethanol gave m.p.207.5°(decomp.).

1.1.9. L-Glucose Phenylosazone.

A solution of the mixture of L-glucose and L-mannose (8g.) in water (100ml.), obtained through the nitroalcohol from L-arabinose, by the method of Sowden and Fischer (1947), was warmed on the boiling water bath. A hot solution of phenylhydrazine (12ml.) in glacial acetic acid (8ml.) was added, together with sodium acetate (2g.) and the mixture heated for 90 minutes. The mixture was cooled and filtered, and the residue washed with 2N-acetic acid, water, and ethanol, giving dark yellow crystals (5.1g.). After treatment with charcoal and recrystallisation from absolute ethanol L-glucose phenylosazone (4.7g., 31%) m.p. 204-205° (decomp.) was obtained. Mixed m.p. with D-glucose phenylosazone (m.p. 208°) gave m.p.191-193°.

1.1.10. D-Glucose-6-Phosphate Phenylosazone.

The barium was precipitated from barium-D-glucose-6-phosphate (2g.) in water (10ml.) by addition of dilute sulphuric acid. After removal of the barium sulphate, glacial acetic acid (1.5ml.) was added to the solution of D-glucose-6-phosphoric acid, and the mixture heated on the

boiling water bath. A hot solution of phenyl hydrazine (3ml.) in 2N-acetic acid (10ml.) was added, with stirring. The phenylosazone separated out in 3 minutes as an orange coloured precipitate. The mixture was heated for the further 15 minutes, cooled and filtered. The precipitate was washed with 2N-acetic acid, water, and ethanol-ether (2:3). After drying in a desiccator the amorphous orange-powder of the phenylosazone of D-glucose-6-phosphoric acid gave m.p.142-146° (decomp.). Attempts to recrystallise the material from alcohol were unsuccessful.

1.1.11. D-Galactose Phenylosazone.

The osazone was prepared from D-galactose by a procedure identical with that used for the preparation of D-glucose phenylosazone (1.1.8.). The product, recrystallised from absolute alcohol, gave D-galactose phenylosazone in 67% yield, m.p.184-186°.

1.1.12. Diphenyl Phosphoryl Chloride.

Crystalline phenol (50g.) was mixed with phosphorus oxychloride (50g.), and heated under reflux for 5½ hours to a temperature of 180°, after the method of Brigl and Muller (1939). The heating was continued for a further 45 minutes to a temperature of 260°. Fractional distillation gave (i) phenyl phosphoryl dichloride (10g., b.p.130-154°, 0.3mm.), (ii) diphenyl phosphoryl chloride (35g., b.p. 154-165°, 0.3mm.) and (iii.) triphenyl phosphate (20g.,

b.p.165-175°, 0.3mm.). The triphenyl phosphate crystallised out on standing.

Diphenyl phosphoryl chloride was obtained as a colourless liquid, with a characteristic pungent odour. It was stored in a stoppered flask, protected from sunlight, at room temperature. Under these conditions it retained its phosphorylative power for 3 years.

1.1.13. Silver Diphenyl Phosphoryl Chloride.

Diphenyl phosphoryl chloride (7.5g.) was heated with 2.5N-sodium hydroxide (20ml.) and silver diphenyl phosphoryl chloride precipitated by adding 10% silver nitrate (85ml.), according to the method of Posternak (1949). The product (9.1g., 96%) was recrystallised from water in fine white needles.

1.1.14. Tri-O-isoPropylidene-D-Glucosone Hydrate.

The compound was prepared by the method of Fewster (1953). Fructose (90g.) was dissolved in methanol (2000ml.) and heated on the boiling water bath. When the solution had reached boiling point cupric acetate (400g.) was added cautiously, and the mixture was refluxed for 15 minutes. The precipitate of cuprous oxide was filtered off and washed with methanol, and copper precipitated from the filtrate and washings with hydrogen sulphide. After removal of the copper sulphide the methanolic extract was distilled

under reduced pressure to give a thick, light brown syrup (88g.). The syrup was dissolved in acetone (2000ml.), to which had been added concentrated sulphuric acid, (62ml.), and the mixture was shaken for 18 hours. The solution was neutralised, with cooling, with solid sodium carbonate, and after filtration was evaporated under reduced pressure to a brown semi-crystalline syrup. The syrup was extracted with hot water (thrice) to remove isopropylidene derivatives of fructose, dissolved in methanol, and water added to incipient cloudiness. Crystals of tri-O-isopropylidene D-glucosone hydrate (4.4g., 2.8%) m.p.120-121° were obtained, which on recrystallisation from methanol gave m.p.123-124°. A mixed m.p. with an authentic specimen of the tri-isopropylidene derivative (m.p.125°) gave m.p.123-124°.

1.1.15. Di-O-isoPropylidene-D-Glucosone hydrate.

The tri-isopropylidene derivative of D-glucosone (2.5g.) was dissolved in 85% acetic acid (50ml.), and the mixture maintained at 50° during 8½ hours, and at 0° for 18 hours. The mixture was evaporated under reduced pressure at 40° to a pale yellow syrup. Residual acetic acid was removed by codistillation with toluene with reduced pressure. A pale yellow syrup of di-O-isopropylidene-D-glucosone hydrate (1.9g., 80%), was obtained.

1.1.16. Di-O-acetyl-di-O-isoPropylidene-D-Glucosone Hydrate.

Di-O-isopropylidene-D-glucosone hydrate (1.9g.), was dissolved in acetic anhydride (20ml.) and warmed at 70° for 5½ hours with anhydrous sodium acetate (0.5g.). A few crystals of ice were added to the cooled mixture which, after 30 minutes, was poured into ice water (100ml.). After adding solid sodium carbonate to pH 6 the mixture was extracted with chloroform. The dried chloroform extract ($MgSO_4$) was evaporated to a syrup under reduced pressure and codistilled with toluene to remove traces of acetic acid. The syrup was crystallised from methanol-water giving di-O-acetyl-di-O-isopropylidene-D-glucosone hydrate (1.82g. 77%), m.p. 69-70°, $[\alpha]_D^{18}$ = + 16.0° (c, 2.0 in ethanol).

1.2. Sugars.

1.2.1. 2-Deoxy-D-Glucose.

Triacetyl-D-glucal was prepared from tetra-O-acetyl-α-D-glucopyranosyl bromide by an adaptation of the method of Fischer (1914) and deacetylated with methanol containing a trace of sodium (Overend, Stacey, and Stanek, 1949). Crystalline 2-deoxy-D-glucose was obtained after treatment of the D-glucal syrup with dilute sulphuric acid (Bergmann, Schotte, and Lechinsky, 1922).

Cramer (1952) prepared the deoxy sugar by this same general method, which is similar to that described by Overend *et al* (1949).

Zinc dust (100g.), 50% sodium acetate solution (200ml.) and 60% copper sulphate solution (20ml.) were mixed with cooling. Glacial acetic acid (250ml.) was added and the mixture cooled to 2-4°. A solution of tetra-O-acetyl-α-D-glucopyranosyl bromide (50g.) in glacial acetic acid (250ml.) was added, with cooling, over a period of 90 minutes. After filtering off the zinc in a charcoal covered filter, water (300ml.) was added to the filtrate. Care was taken that the zinc did not dry completely, as it is pyrogenic under these conditions. The benzene extracts (150ml., thrice) of the aqueous solution were washed with water (twice),

sodium hydrogen carbonate solution (twice) and water, and finally dried (Na_2SO_4). Benzene was removed under reduced pressure, final traces being removed by codistillation with ethanol under reduced pressure. Triacetyl-D-glucal (28.0g., 85%) was obtained as a clear white syrup which crystallised spontaneously after removeal of all traces of alcohol, m.p.49.9-51.0°. $[\alpha]_D^{18}$ = -12.8°., c,(2.0 in alcohol). Attempts at further recrystallisation from alcohol, or ether/pet. ether were unsuccessful.

Triacetyl glucal (25g.) dissolved in methanol (100ml.) was heated to boiling on a water bath, and made alkaline to phenolphthalein with 0.1N-sodium methylate (7ml.) over a period of 25 minutes. After cooling quickly to -10°, the methanol was removed under reduced pressure and glucal (12.0g., 89%) was obtained as a clear colourless syrup. Unsuccessful attempts were made to crystallise the product from ethyl acetate.

Glucal syrup (10.0g.) was taken up in water (100ml.) containing 7.5N-sulphuric acid (2ml.) and left at room temperature during 18 hours. Solid barium carbonate and aqueous barium hydroxide were added to neutrality, the mixture heated to 40° and shaken for an hour. Charcoal (2g.) was added and the mixture filtered. The clear filtrate was evaporated under reduced pressure to give a clear colourless syrup (7.5g.) The syrup was dissolved in dry isopropyl

alcohol (50ml.), which was removed under reduced pressure, this procedure being repeated twice. On allowing the dry syrup (7.0g.), dissolved in dry isopropyl alcohol (30ml.), to stand at room temperature, small, white crystals of 2-deoxy-D-glucose (3.7g., 33%) were obtained. Recrystallisation from isopropyl alcohol gave m.p. 144-145°, $[\alpha]_D^{17}$ = +38.4 → +44.8 in 40 minutes (c, 1.0 in water). $[\alpha]_D^{17}$ = +16.0 → +87.6 in 22 hours (c, 1.0 in pyridine). The α methyl phenylhydrazone of 2-deoxy-D-glucose, obtained in pure white needles gave m.p.154-156° on recrystallisation from 90% ethanol.

1.2.2. 1:5-Anhydro-D-Glucitol.

The compound was prepared after the method of Ness, Fletcher and Hudson (1950).

A solution of tetra-O-acetyl-D-glucopyranosyl bromide (10g.) in diethyl ether (200ml.) was added dropwise, with stirring, to a suspension of lithium aluminium hydride (10g.) in diethyl ether (200ml.). The temperature was kept at 30-35° during the 50 minute addition period. Water (175ml.) was cautiously added to the mixture, which was filtered to remove aluminium hydroxide. The washed precipitate and the ether layer were discarded, and the aqueous solution deionised by passage through the ion exchangers, Amberlite I.R. 120 and Amberlite I.R. 4B. The deionised extract was evaporated under reduced pressure at 40-60° to give a pale yellow

crystalline mass (3.0g., 76%). Recrystallisation from absolute alcohol gave 1:5-anhydro-D-glucitol (2.5g.) in small white crystals m.p.139-141°, $[\alpha]_D^{17}$ = +42.0 (c, 1.0 in water).

1.2.3. 1:5-Anhydro-Mannitol.

The substance was prepared from tetra-O-acetyl-D-mannopyranosyl bromide by a method identical with that used in the preparation of 1:5-anhydro-glucitol.

1:5-anhydro-D-mannitol was obtained in poor yield (19%) as small white crystals m.p.153-155°, $[\alpha]_D^{17}$ = -48.8 (c, 1.0 in water).

1.2.4. D-Glucosone.

D-Glucose phenylosazone (10g.) was triturated with 96% ethanol (300ml.) and poured into a 2-litre three-necked flask fitted with a dropping funnel, stirrer, and condenser. Glacial acetic acid (6ml.), benzaldehyde (16ml.), and distilled water (500ml.) were added. After refluxing for 2½ hours with constant stirring the solution became clear; refluxing was continued for a further 2 hours, during which benzylidene phenylhydrazone separated out. With the condenser reversed 200ml. of distillate were collected in 30-45 minutes, distilled water (500ml.) being added concurrently through the dropping funnel. The reaction products were syphoned from the flask, allowed to cool overnight and the precipitated

benzylidene phenylhydrazone filtered off. The combined filtrate and washings were evaporated under reduced pressure at 40° to a volume of less than 200ml. and extracted 5-6 times with ether (200ml.) to remove residual benzylidene phenylhydrazone, benzoic acid etc. After treatment with charcoal a yellow solution was obtained which on evaporation in vacuo at 40° yielded D-glucosone as a pale yellow syrup (2.6g.). The syrup was dissolved in water (8ml.) at 40° and extracted with hot 96% ethanol (300ml.) and after a further treatment with charcoal the solution was evaporated to a syrup. The syrup was dissolved in methanol (30ml.) and evaporated to dryness under reduced pressure to give a light yellow "froth" of D-glucosone (2.3g., 46%). A pure white froth could be obtained by keeping the yellow material for several weeks at 0°, and treating with charcoal. The osone reduced Fehling's solution in the cold and gave a blue colour with Benedict's arsenophosphotungstic acid reagent for uric acid in the presence of alkali cyanide. With phenylhydrazine in acetic acid, D-glucose phenylosazone was rapidly formed at room temperature.

1.2.5. <u>L-Glucosone.</u>

L-glucose phenylosazone (4.5g.) was treated with benzaldehyde (7.0ml.) in the manner described for the preparation of D-glucosone (1.2.4.). L-glucosone was obtained in 40% yield, and gave L-glucose phenylosazone m.p.203-204°

when treated with phenylhydrazine and acetic acid. L-glucosone was chromatographically identical with D-glucosone in phenol/water and saturated butanol/acetic acid.

1.2.6. D-Galactosone.

D-Galactosone was prepared from D-galactose phenylhydrazone by the method used for the preparation of D-glucosone, and was obtained in 40% yield. It showed the same chemical properties as D-glucosone. D-galactose phenylhydrazone m.p.183-184° was obtained by treatment of D-galactosone with phenylhydrazine and acetic acid.

1.3. Sugar Phosphates.

1.3.1. D-Glucose-6-Phosphoric acid.

(a) 1:2-O-isopropylidene-D-glucose was tosylated after the method of Meyer and Reichstein (1946).

Freshly crystallised p-toluene-sulphonyl-chloride (20g.) in alcohol-free chloroform (100ml.) at 5° was added to 1:2-O-isopropylidene-D-gludose (25g.) in freshly distilled pyridine (75ml.), the temperature being maintained at 5-10°. After standing at 21° for 18 hours 5% hydrochloric acid (300ml.) was added to the mixture, which was then extracted with ether (100ml. 8 times). The ether extract was washed with 5% hydrochloric acid (twice), 5% sodium carbonate (twice) and water (twice). Reduction of the extract under reduced pressure afforded a syrup (20g.) from which pure white crystals of 1:2-O-isopropylidene-6-O-toluene-p-sulphonyl-D-glucose (12.0g., 28%), m.p.95-97° were obtained. Recrystallisation from ether/pet. ether gave m.p.98-99°.

The tosylated derivative was treated with sodium methylate in the cold, according to the method of Ohle and Tessmar (1938) to give 1:2-O-isopropylidene-5:6-anhydro-D-glucose in 74% yield, m.p.130-131.5°.

1:2-O-isopropylidene-5:6-anhydro-D-glucose (1.0g.) was added to dipotassium hydrogen phosphate (1.62g.) in water (30ml.), and the mixture heated under reflux for 32 hours.

Regenerated cation exchange resin Amberlite I.R. 120 (5g.) in the acid form, was added, and the refluxing continued for 1 hour. After removal of the resin by filtration, the solution was evaporated under reduced pressure to 10ml. and neutralised with potassium hydroxide. Potassium glucose-6-phosphate precipitated on addition of 4 volumes of methyl alcohol, was separated off and washed with methyl alcohol, methyl alcohol/ether and ether. The dried amorphous white, potassium glucose-6-phosphate (1.31g., 78%) was dissolved in water (5ml.) and the potassium removed with Amberlite I.R. 120. The solution was neutralised to pH 8 with aqueous barium hydroxide, and the sugar phosphate precipitated with 4 volumes of ethyl alcohol. The dried precipitate was an amorphous white powder of barium glucose-6-phosphate (1.32g., 67%), $[\alpha]_D^{16}$ = +12.2 (c, 5.0 in water). (Found: P, 7.0: Ba, 29.8%. Calc. for $C_6H_{11}O_9PBa.2H_2O$: P, 7.2; Ba, 31.8%.).

1.3.1.

(b) A solution of 1:2-O-iso-propylidene-O-3:5-benzylidene-D-glucose (2.5g.) in pyridine (4ml.) was cooled to 0° and treated with diphenyl phosphoryl chloride during 10 minutes at 0°, and then at room temperature overnight. A few crystals of ice were added to the mixture which was poured, after 10 minutes, into ice water (50ml.). The product was thrown down as a pasty mass which was extracted with ether.

The ether extract was washed with water (twice), 0.5N-sulphuric acid (thrice), water, and dilute aqueous sodium hydrogen carbonate. After being dried ($MgSO_4$) the solvent was evaporated and diphenyl(1:2-O-isopropylidene-O-3:5-benzylidene-D-glucose-6)-phosphate (3.4g., 78%) was obtained as a colourless syrup.

A solution of the diphenyl phosphate (3.4g.) in ethanol (25ml.) and water (1ml.) was freed from catalyst poisons by boiling under reflux with Norite (1g.) for 10 minutes. The combined filtrate and washings were shaken with hydrogen at slight over-pressure with Adams' platinum oxide (100mg.). Absorption of hydrogen (100ml.) was complete in 12 hours. The solution was filtered from the catalyst and evaporated to dryness under reduced pressure. 1:2-O-isopropylidene-O-3:5-benzilidene-D-glucose-6-phosphoric acid (2.2g., 90%) was obtained as a colourless syrup and was hydrolysed in water (50ml.) containing N-sulphuric acid (2ml.) for 50 minutes at 90°. After this time the compound went into solution. The solution was neutralised to pH 8 with aqueous barium hydroxide, the insoluble barium residues were collected and washed with water, and the combined washings and filtrate were treated with 2 volumes of acetone. The floculent precipitate was collected by centrifugation, and was washed with acetone, acetone/ether, and ether. The

white powder obtained on drying was dissolved in water (4ml.) and 2 volumes of acetone added. The precipitate was centrifuged down and washed as before. Barium D-glucose-6-phosphate (0.90g., 40%) was obtained as a white powder, being completely soluble in water and strongly reducing $[\alpha]_D^{16} = +12.0.$, (c, 5.0 in water). (Found: P, 6.9; Ba, 31.4%. Calc. for $C_6H_{11}O_9PBa.2H_2O$: P, 7.2; Ba, 31.8%).

1.3.2. D-Fructose-1-phosphoric Acid.

A solution of 2:3-4:6-di-O-isopropylidene-D-fructose (2.0g.) in dry pyridine (3.0ml.) was treated with diphenyl chlorophosphonate (2.45g.) in a manner identical with that used in the preparation of diphenyl(1:2-O-isopropylidene-O-3:5-benzylidene-D-glucose-6)phosphate (1.3.1.b.). From the crude product diphenyl (2:3-4:6-di-O-isopropylidene-D-fructose-1) phosphate (3.1g., 86.0%) was obtained as a faintly yellow syrup.

The syrup (2.5g.) was dissolved in methanol (50ml.) and water (1ml.) and hydrogenated by the method described previously (1.3.1.b.). Hydrogen (700ml., 7.0mols.) was absorbed during 6½ hours. Filtration and evaporation gave 2:3-4:6-di-O-isopropylidene-D-fructose-1-phosphate (1.5g. 90.0%) as a pale yellow syrup.

This syrup (1.5g.) in water (15ml.) was heated on a steam bath for 40 minutes. Inorganic phosphorus was filtered off after neutralisation with barium hydroxide

to pH 8.0. Addition of 2 volumes of acetone precipitated barium D-fructose-1-phosphate, which was centrifuged off after standing overnight at 0°. The barium salt was dissolved in water (10ml.) and precipitated with 2 volumes of acetone. After a repetition of this procedure the barium salt of D-fructose-1-phosphoric acid (.78g.) was obtained as a white amorphous powder completely soluble in water.

$[\alpha]_D^{16} = -32°$ (c,1.8 in water). (Found: P, 7.0; Ba, 30.2% Calc. for $C_6H_{11}O_9PBa.2H_2O$: P, 7.2; Ba, 31.8%.).

1.3.3. Attempted Preparation of D-Glucosone-6-Phosphoric Acid.

Attempts were made to prepare this ester by three different methods. (a) by a modification of the method of von Lebedew (1910) who treated sodium-D-glucose-6-phosphate phenylosazone with benzaldehyde by the method of Fischer and Armstrong (1902); (b) by phosphorylation of di-O-isopropylidene-D-glucosone hydrate using diphenylphosphoryl chloride, and (c) by treatment of the di-O-isopropylidene-5:6-anhydro derivative of glucosone with dipotassium hydrogen phosphate.

1.3.3.

(a) The phenyl osazone of glucose-6-phosphoric acid (1.5g.) was neutralised with 5N-sodium hydroxide and treated with benzaldehyde by a method identical with that used for D-glucosone (1.2.4.). A yellow syrup (0.4g.) was obtained which contained no phosphorus but gave a blue colour with

Benedict's arsenophosphotungstic reagent for uric acid in the presence of alkali-cyanide.

1.3.3.

(b) Di-O-acetyl-di-O-isopropylidene-D-glucosone hydrate, (0.5g.) was refluxed with 0.1N-sodium hydroxide (50ml.) for 2 hours. The solution was cooled and neutralised to pH 8.5. The cooled mixture was extracted with chloroform and the extract dried over sodium sulphate. Evaporation under reduced pressure at 40° afforded di-O-isopropylidene-D-glucosone hydrate (0.33g. 94%) as a colourless syrup.

This syrup (0.33g.) was dissolved in ice-cold pyridine (2ml.) and diphenyl phosphoryl chloride (0.38g.) was added dropwise in the cold. After 5 minutes at 0° the mixture was left overnight at 16°. A few crystals of ice were added to the mixture which was poured, after 10 minutes, into ice-water (12ml.). The ether extract of this mixture was washed with water, sulphuric acid, water and dilute aqueous sodium hydrogen carbonate, and finally dried over magnesium sulphate. Filtration and evaporation afforded a colourless syrup which was not characterised. After charcoal treatment an alcoholic solution of the syrup was treated with hydrogen in the presence of Adam's platinum oxide for 8 hours. No hydrogen was adsorbed and the original syrup (0.07g.) was recovered.

1.3.3.

(c) Di-O-*iso*propylidene-D-glucosone hydrate (0.35g.) obtained through the diacetyl compound as in 1.3.3.b. was dissolved in alcohol-free acetone (3.5ml.) and pyridine (0.2ml.) added. The mixture was cooled in an ice-salt mixture and freshly purified *p*-toluenesulphonyl chloride (0.29g.) was added with constant shaking. After remaining at -3° for 10 minutes the mixture was left at 0° for 6 hours. A few crystals of ice were added to the solution and after 30 minutes, ice-water (10ml.) was added. The mixture was extracted with chloroform, and the extract washed and dried ($MgSO_4$). After filtration and evaporation a white syrup (0.32g.) was obtained. The syrup was dissolved in acetic anhydride (4.0ml.) and warmed at 70° for 5 hours with anhydrous sodium acetate (0.1g.). The cooled reaction mixture was poured into ice-water (20ml.) and adjusted, with stirring, to pH 6 with sodium bicarbonate. The dried chloroform extract was evaporated to a syrup under reduced pressure and acetic acid was removed by codistillation with toluene. The syrup was crystallised with difficulty from ether/pet. ether (b.p.40-60°) giving a mixture of substances having m.p.56-58°, and 67-68°. The components of the mixture were not separated and attempts were made to tosylate the di-*iso*propylidene derivative by a more vigorous treatment.

Di-O-*iso*propylidene-D-glucosone hydrate (0.3g.)

in alcohol-free acetone and dry pyridine (0.2ml.) was treated with p-toluenesulphonyl chloride for 30 minutes at 0° and 18 hours at 20°. The tosyl derivative isolated as before, was obtained as a pale yellow syrup (0.25g.) which could not be crystallised. The syrup (0.25g.) in dry methanol (4ml.) was treated in the cold for 15 minutes with N-sodium methylate. The mixture was poured into water and extracted with chloroform. The dried extract ($MgSO_4$) afforded a pale yellow syrup (0.16g.). This syrup was treated with dipotassium hydrogen phosphate in the manner described in 1.3.1.a., and a barium salt (0.03g.) obtained finally, by methanol precipitation from water. A solution of the salt gave a blue colour with Benedict's arsenophosphotungstic reagent in the presence of alkali cyanide. The compound could not be identified chromatographically.

2. The Isolation of Biologically Prepared Compounds.

2.1. Co-enzyme I. - diphosphopyridine nucleotide - DPN.

DPN was extracted from yeast by three different methods, and the crude product partially purified by ion-exchange chromatography.

2.1.1. Extraction of the Crude Material.

(a) After the method of Sumner, Krishnan and Sisler (1947).

Fresh bakers' yeast (454g.) was mixed with diethyl ether (350ml.). A cooled mixture of 96% ethanol (350ml.) and concentrated sulphuric acid (22g.) was added to the yeast-ether mixture. After stirring for 10 minutes a clear brown solution was filtered from the pasty mass. 2 volumes of ethanol were added to the filtrate, and the formed precipitate centrifuged off, washed with absolute ethanol (twice) and ether, to give DPN (2.6g.) assaying 8% purity.

2.1.1.

(b) After the method of Clark, Dounce and Stotz (1949).

Fresh bakers' yeast (5 lb.) was crumbled into cold acetone (1750ml.), and a mixture of acetone (1750ml.) and concentrated sulphuric acid (270ml.) added. After stirring for 15 minutes the mixture was filtered. One volume of ethanol was added to the filtrate, and the precipitate

formed was filtered off and discarded, being mainly potassium hydrogen sulphate. The addition of a second volume of ethanol precipitated the nucleotide, which was centrifuged off and 3 times precipitated from water by ethanol. The dried DPN (1.6g.) assayed 21%.

2.1.1.

(c) Fresh bakers' yeast (5 lb.) was crumbled into hot water (2500ml.) kept at 90°. The heating was continued while hyflo super cel (20g.) was added and the mixture was then cooled rapidly. DPN (1.2g.) was isolated in a manner identical with that used in 1.1.2. with a purity of 16%.

2.1.2. Purification of Crude DPN.

A column (2.5 x 32.0cms.) of Amberlite I.R.400 ion-exchange resin was treated with 5% HCl (2000ml.), water (2000ml.), 5% NaOH (2000ml.), water (4000ml.) and finally 0.1M ammonium acetate-acetic acid buffer of pH 5.9 (3000ml.). Crude DPN (3.0g.) was dissolved in 0.1M ammonium acetate (30ml.) and poured onto the prepared column. Elution was carried out with 0.1M ammonium acetate buffer at a flow rate of 1ml./minute. The eluate was collected in 10ml. fractions and the DPN content of the fractions after acidification was determined by measuring the optical density at 340mμ. Fractions 8 to 20 (80-200ml.) which gave high absorption at 340mμ were combined and cooled to 0°. 10% nitric acid was added to

pH 2.5, and the nucleotide precipitated by addition of 3 volumes of acetone. The precipitate was centrifuged off, washed with absolute alcohol and ether, and dried. DPN (0.4g.) was obtained as a white powder of 74% purity and was kept over P_2O_5 in a desiccator.

2.2. Barium Adenosine Triphosphate.

The barium salt of ATP was isolated from rabbit muscle by a combination of the methods of Kerr (1941) and Dounce, Rothstein, Beyer, Meier, and Freer (1948).

A rabbit was killed by a blow on the neck, and the muscle (500g.) quickly dissected off, minced and extracted twice with 10% trichloroacetic acid (300ml.). The combined extracts were filtered and neutralised with 10% NaOH (100ml.) to pH 7.3. Glacial acetic acid (3.0ml.) was added so that final acetate concentration was 0.2%, and 20% mercuric acetate (70ml.) in 2% acetic acid was added slowly, with stirring. The precipitated nucleotide was centrifuged off, suspended in water (150ml.) and treated with hydrogen sulphide. After removal of the precipitated mercuric sulphide the filtrate and washings were aerated to remove traces of hydrogen sulphide. The solution was adjusted to pH 7 by addition of 10% sodium hydroxide solution, and 2M-barium acetate solution (5ml., i.e. 1ml. per 100g. of the original muscle) was added.

The precipitate of water insoluble barium nucleotides was allowed to settle overnight at 0^{o} and removed by centrifugation. The barium nucleotide after being washed with 2 volumes of ice water was dissolved in the minimal amount of glacial acetic acid. The nucleotide solution was diluted to 500ml. and the formation and decomposition of the mercuric salt carried out as described previously. The hydrogen-

sulphide-free solution was adjusted to pH 7 by the addition of 10% sodium hydroxide, and 2M-barium acetate (5ml.) was added. The precipitate was allowed to settle overnight, centrifuged off and washed with water, 50% ethanol, 75% ethanol, 96% ethanol and ether. The white amorphous material was dried over sulphuric acid, pulverised and stored over sulphuric acid in vacuo.

0.75 to 1.00g. of barium adenosine triphosphate was obtained per rabbit. Higher yields are possible when the rabbit is anaesthetized by intraperitoneal injections of 25% magnesium sulphate, or nembutal, but providing the rabbit is kept in a quiet state with relaxed muscles prior to its despatch, reasonable yields of Ba ATP can be obtained without anaesthesia.

(Found: N (Kjeldahl), 8.05; total organic P, 11.08; hydrolysable P, 7.12; ba, 32.31. Calc. for $Ba_2(C_{10}H_{12}O_{13}N_5P_3).4H_2O$: N, 8.2; total organic P, 10.88; hydrolysable P, 7.26; Ba, 32.17%. Ratio of total organic P to hydrolysable P (7mins. at 100° in 1.0N-HCL): found, 1.57; calc. 1.5%.).

There was 0.6% inorganic phosphorus present in the preparation, probably as barium phosphate. The high ratio of total organic P: 7mins. hydrolysable P suggests that ADP was also present.

The preliminary phosphorylation experiments were carried out using the ATP prepared by the present author. ATP obtained commercially (Light & Co.) was used for the majority of the experiments. The purity of the samples used varied from 65-75% for the free acid, and 90-95% for the sodium salt of the acid.

2.3. D-Glucose-1-Phosphoric Acid.

Potato starch was broken down by potato phosphorylase in the presence of a phosphate buffer. After deproteinisation by heating, and precipitation of the excess phosphate with magnesium acetate, glucose-1-phosphoric acid was isolated using ion exchange columns. The method used follows that described by McCready and Hassid (1944).

The opaque brown coloured juice (450ml.) obtained by macerating fresh, peeled potatoes, (1000g.) in a Waring blender, was clarified by filtering through Hyflo Super Cell. Potato starch (50g.) was stirred into cold water (300ml.) and then poured into boiling water (1500ml.), and the mixture boiled for 30 minutes. A phosphate solution of pH 6.7 was made by dissolving sodium dihydrogen phosphate monohydrate (69g.) and disodium hydrogen phosphate duodeca hydrate (179g.) in water (1500ml.).

The potato juice was added to the mixed starch and phosphate solutions, and the mixture was allowed to stand at 37° for 24 hours. The solution (3400ml.) obtained after removal of protein and phosphate from the incubation mixture was passed through a column of the cation exchange resin I.R. 120, and then through a column (7 x 30cm.) of the anion exchanger, I.R. 4B, at pH7.

Glucose-1-phosphoric acid was eluted off the basic resin with 4% ammonium hydroxide (350ml.) at a flow rate

of 2ml. per minute. Elution was considered to be complete when the eluate reached pH 11. The eluate was treated with potassium acetate (30g.) and 10% potassium hydroxide added to pH 12. On remaining overnight at 0° after addition of 2 volumes of methanol, a white crystalline material was obtained. Filtration afforded white crystals of hydrated dipotassium D-glucose-1-phosphoric acid (10.3g.) which were washed with methanol and ether, and dried over P_2O_5.

$[\alpha]_D^{16} = +76.8^\circ$ (c, 2.0 in water). (Found: Organic P, 8.25. Calc. for $C_6H_{11}O_9PK_2.2H_2O$: organic P, 8.38).

There was present 1.7% of inorganic phosphate but on the basis of the organic phosphate estimation the dipotassium glucose-1-phosphate was of 98.2% purity.

2.4. D-Glucose-6-Phosphoric Acid.

The ester was prepared by the method of Swanson (1950). A solution of starch was incubated with a mixture of potato juice and crude phosphoglucomutase, obtained from rabbit muscle. D-glucose-6-phosphoric acid was isolated from the deproteinised mixture as the barium salt (5.2g.). The salt was purified by repeated precipitation from water with alcohol. Barium D-glucose-6-phosphate (3.1g.) was finally obtained as a fine white powder, completely soluble in water, having strong reducing properties. $[\alpha]_D^{16} = +11.6$, (c, 5.0 in water). (Found: Ba, 29.0; P, 6.6%. Calc. for $C_6H_{11}O_9PBa.2H_2O$: Ba, 31.8; P, 7.2%.).

The material was free of inorganic phosphate.

2.5. Fructose-1:6-diphosphoric Acid.

2.5.1. Preparation of the Crude Material.

Sucrose (200g.), dissolved in a solution of sodium dihydrogen phosphate (42g.) and sodium hydrogen carbonate (11g.) in water (1000ml.), was fermented with bakers' yeast (450g.) in the presence of ether (150ml.), according to the method of Neuberg and Lustig (1942a). The mixture was boiled after 9 hours, to coagulate the protein which was removed by filtration. Hexose phosphates were isolated from the filtrate (1) using two methods.

2.5.2. Isolation of the Pure Product.

(a) The filtrate was neutralised to pH 8.5 with 4N-sodium hydroxide and 55% calcium chloride solution (100ml.) was added. The mixture was heated on the boiling water bath for 20 mins. to complete precipitation, and the warm solution was filtered. A brown, amorphous precipitate of crude calcium fructose-1:6-diphosphate (18g.) was obtained. The precipitate was dissolved in 0.5N-hydrochloric acid (150ml.), filtered and passed through a column (7 x 32cm.) of the cation exchange resin I.R. 120. The eluate was made to pH 8.0 by the addition of 2N-sodium hydroxide, and 2M-barium acetate (10ml.) was added. The white, flocculent precipitate formed, was centrifuged off and dried over P_2O_5. Barium fructose diphosphate (8.3g.) was obtained containing

5.4% of inorganic phosphorus. This barium salt was purified by the method following, to give barium fructose diphosphate (6.4g.) of 96% purity.

2.5.2.

(b) Magnesium acetate tetrahydrate (40g.) was added to the filtrate (1), followed by concentrated ammonia solution to pH 8.5. The precipitated inorganic phosphate was filtered off, and the filtrate was passed through the cation exchange resin I.R. 120, and the anion exchange resin I.R. 4B. Fructose diphosphoric acid was eluted off the basic column with 4% ammonium hydroxide (400ml.) at a flow rate of 1ml. per minute. The addition of barium acetate to the eluate gave a flocculent, white precipitate which was centrifuged off, washed with water, 95% ethanol (twice) and ether, and dried. Barium fructose diphosphate (2.3g.) was obtained as a white amorphous powder of 96% purity. (Found: total organic P, 9.8; Ba, 44.5%. Calc. for $C_6H_{10}O_{12}P_2Ba_2$: total organic P, 10.2; Ba, 44.9%.).

3. Yeast Preparations.

Actively fermenting cell-free extracts of bakers' yeast are not readily obtainable by the methods used by Buchner (1897) and Lebedew (1911) for the preparation of active extracts from brewers' yeast. Some modifications of their original methods were investigated and cell-free preparations of bakers' yeast obtained which fermented glucose or fructose diphosphate. An active cell-free extract was also obtained by a controlled cold-treatment of bakers' yeast.

3.1. Disintegrative Methods.

The mechanical disruption of yeast cells was attempted by grinding, or shaking a thick yeast suspension with steel ball bearings or Pyrex glass powder or Ballotini (0.1mm. glass beads), or mixtures of these materials. The grinding or shaking was carried out for varying periods of time, up to 5 hours. A small amount of cell disruption occurred only after the most vigorous treatment but no enzymically active extracts were obtained.

An acetone-dried cell-free preparation which fermented glucose was obtained by an adaptation of the method of Hochster and Quastel (1951). Fresh bakers' yeast (10g.) was mixed with nicotinamide (0.5g.), Ballotini (5.0g.) fine Pyrex glass powder (10g.) and 0.02M potassium hydrogen phosphate (5ml.). The Pyrex powder was prepared by grinding

Pyrex glass in a mortar and taking off the finest particles by flotation from a beaker having a slow running stream of water. The mixture was homogenised, in the cold, in a glass Potter-Elvehjem homogeniser, for three 5 minute periods, at 400r.p.m. The cell-free supernatent obtained on centrifuging off the cell debris and glass powder was poured into cold acetone. The flocculent precipitate was filtered off and washed quickly with cold, dry acetone. A white powder (500mg.) was obtained which had fermentative ability, but no preparations were obtained having an activity as high as that reported by Hochster and Quastel (1951).

3.2. Maceration Juice.

Maceration of dried bakers' yeast, with water, does not produce enzymically active juices, but Neuberg and Lustig (1942b) reported that active juices could be obtained by macerating American bakers' yeast with ammonium phosphate. An active fermenting juice was obtained from DCL bakers' yeast by an adaptation of this method, using a more vigorous treatment.

Fresh bakers' ~~yeast~~ (450g.) was crumbled into small fragments and spread in 3-5mm. layers on large sheets of filter paper. The yeast was dried in air at 18-20° for 84 hours. The hard, light-brown granules of dried yeast were ground to a powder and stored in a stoppered bottle at 0°. This dried yeast powder was macerated with 3 volumes of ammonium hydrogen phosphate for 6 hours at 37° and then shaken vigorously for 3 hours at 37°. The clear, yellow liquid which was centrifuged off from the maceration mixture, retained its fermentative ability for 8-10 days when stored at 0° with a drop of toluene. Active maceration juices were obtained from the dried powder which had been stored at 0° for 3 months.

The maceration juice obtained in this manner fermentated glucose only after an induction period of 50 minutes. The addition of an activating mixture containing 8×10^{-5}M ATP, 2.0×10^{-6}M DPN, 2×10^{-4}M Mg^{++}, 2×10^{-4}M

Mn^{++}, and 0.125M acetaldehyde decreased this induction period by 40-45 minutes.

Fructose diphosphate was fermented readily in the absence of activators after an induction period of about 5-10 minutes.

3.3. Dry Ice Yeast.

3.3.1. Introduction.

The acetone dried extracts and the Lebedew extract (3.1., 3.2.) suffer from the defect that not all of the enzymes present in the intact cell are found in the corresponding extract. Frozen extracts, on the other hand, contain enzymes which are normally left behind with the cell fragments. The first frozen extracts were prepared by Dixon and Atkins (1913), by placing pressed brewers' yeast, wrapped in paper, in liquid air for 10-15 minutes. On allowing the frozen yeast to thaw out, a liquid yeast was produced which had high fermentative ability. It was found that no autolysis took place during this procedure, and that the extract could be concentrated to dryness without damage to the enzymes present. The pH of such extracts was reported to be 6.2, by Tait and Fletcher (1926), which is in good agreement with the pH of the contents of the intact cell reported by Conway and Downey (1950).

Later, Lynen (1939) reported that immersion of fresh, washed, bakers' yeast in liquid air or nitrogen for 2-96 hours destroys respiration and anaerobic fermentative power. The juice obtained from yeast treated in this manner appeared to be identical with Buchner pressed juice, showing all the characteristics of cell-free fermentation. The

juice could be evaporated *in vacuo* below 0°, without change of activity.

Krebs, Gurin, and Eggleston (1952) treated bakers' yeast with an excess of dry ice for 20 minutes, and reported that the liquid yeast obtained, on thawing, could oxidise many di- and tricarboxylic acids which are not affected by untreated yeast cells. The fermentative ability of this preparation was found to be dependent on the initial temperature of the yeast. Yeast which was heated to 50° before cold treatment, showed no fermentative ability whatever.

A liquid yeast prepared by a rather less vigorous treatment than that used by Krebs *et al* had high fermentative ability and phosphorylating activity. The method of preparation and properties of this extract are described below.

3.3.2. Method of Preparation and Properties.

Bakers' yeast was crumbled into a wide-necked polythene bottle which was placed in a solid carbon dioxide-acetone mixture. The bottle was shaken at intervals, and after 15 minutes was placed in a water bath at 20°, when the yeast began to liquify. After three freezing and thawing treatments the yeast became completely liquid. This liquid was the "dry ice" yeast used for experiments described in Part I. 2.3.3.2., and Part II. 2.3.1.

It was found that fresh yeast could be liquified more easily than yeast which had been stored for some time. Yeast, treated immediately after removal from the refrigerator, gave more actively fermenting extracts than yeast treated after remaining at 18° for a few hours. The activities of various cold treated yeast preparations are shown in Table XII.

TABLE XII.

Yeast (25mg. wet/wt.)	Initial Temperature	Fermentation rate µl. CO_2 formed/hour
Fresh	0	400-425
	18	360-380
Stored	0	340-370
	18	320-350

As described in Part I., section 2.3.3.2., the addition of ATP to old dry ice yeast increased the fermentation rate of glucose by these preparations. Freshly prepared cold-treated yeast, in the presence of KF, utilised ATP, measured by the method of Colowick and Kalckar (1943), in the presence of a suitable substrate. Some ATP breakdown occurs without addition of phosphate acceptors, due possibly to the presence of ATPase in the preparation, or endogenous phosphate acceptors. The uptake of ATP was difficult to demonstrate in preparations made from stored yeast, or in old dry ice yeast preparations.

Differential staining with the Gram staining technique showed that about 75% of the cells remined whole after the cold-treatment. Yeast, cultured on agar slants from serially diluted dry-ice yeast showed that 12% of the cells were viable after cold treatment.

3.4. The Isolation of Partially Purified Hexokinase from Bakers' Yeast.

3.4.1. Introduction.

Hexokinase was first obtained in a partially purified form from bakers' yeast by Meyerhof (1927) who, after extracting the toluene-plasmolysed yeast with water, precipitated the enzyme, together with other proteins, with 50% alcohol at 0°. Addition of water to the precipitated protein caused the enzyme to go into solution, leaving much insoluble inactive material.

Colowick and Kalckar (1943) fractionated a hexokinase preparation, obtained by the method described by Meyerhof (1927) with ammonium sulphate. The fraction precipitating between 50 and 75% saturation was dried *in vacuo*, in the cold, and stored in a desiccator. The dry powder was reported to be stable for some months without loss of activity.

Later workers who obtained hexokinase in a highly purified form, prepared an aqueous extract of the enzyme by the method of Meyerhof, but used different techniques to obtain a crystalline product.

Bailey and Webb (1948) found that 1% glucose stabilised the enzyme, confirming van Heyningen's (1941) observation, but found that glucose also retarded autolysis.

Further experiments showed that the addition of cysteine or neutralised sodium sulphide, effected stabilisation and acceleration of autolysis. Further purification was accomplished by adsorption, first of impurities and then the enzyme, on to calcium phosphate gel, final purification and crystallisation being carried out by ammonium sulphate fractionation.

In their method for the isolation of hexokinase Berger et al (1946) used no stabiliser when plasmolysing the yeast, although all subsequent operations were carried out in the presence of 1% glucose. Purification was accomplished by alcohol fractionation and adsorption onto aluminium hydroxide gel. The final crystallisation was carried out, with difficulty, from alcohol, in the cold.

Kunitz and MacDonald's method (1946) for the isolation of the pure enzyme has become the one most generally used. The preliminary plasmolysis was carried out without a stabiliser but all further operations were carried out in the presence of 1% glucose. The methods of Northrop, Kunitz, and others for enzyme crystallisation were then applied to hexokinase. Kunitz and MacDonald were able, by careful ammonium sulphate fractionation, to isolate a concentrated hexokinase fraction which, after fractionation with alcohol yielded crystalline hexokinase.

The three methods described above vary considerably in general, and in detail. It was the purpose of this part of the present investigation to establish a method for obtaining hexokinase in a highly purified, if not crystalline form, by adapting the methods described to the conditions obtaining in this laboratory. These conditions may be summarised:-

(a) The largest convenient amount of yeast which could be plasmolysed at one time was 7 lb.

(b) All larger filtrations had to be done at existing outside, or room temperatures.

(c) Centrifugation could be carried out at temperatures from 5-10° for only very short periods.

Preliminary investigations showed that the conditions suggested by the American workers for the plasmolysis of the yeast were not severe enough to give active hexokinase extracts using the strain of yeast supplied by the DCL Company. Bailey and Webb (1948) also noted the difference between American and English yeasts in this respect, and used long extraction periods in the presence of neutralised sodium sulphide to obtain active aqueous hexokinase extracts. Earlier, Bach, Dixon, and Zerfas (1946), when isolating yeast lactic dehydrogenase, found that Delft yeast was plasmolysed much more readily than Manchester yeast. From a consideration of these findings it was decided to investigate the bast method of yeast plasmolysis for the supplied yeast

It was emphasised by previous workers, and confirmed in the present investigation, that the enzymatic activity of hexokinase is gradually lost at temperatures above 5°, the rate of inactivation increasing rapidly with a rise in temperature. All operations subsequent to the actual plasmolysis were, therefore, carried out at a temperature as near 5° as was practicable. The difficulty in maintaining accurate temperature control at the lower temperatures excluded alcohol precipitation as a method of fractionation of the larger volumes of cruder material.

The investigation, therefore, developed along two main channels, (a) determination of the best method for plasmolysing yeast, and (b) the isolation of an active hexokinase preparation without using alcohol precipitation in the preliminary stages.

3.4.2. Yeast Plasmolysis.

Preliminary experiments were carried out to determine the bast plasmolysing agent for this yeast. The amount of plasmolysis under different conditions was determined by histochemical methods.

The number of yeast cells remaining after plasmolysing treatment was determined by counting the whole cells present in an aliquot of the treated yeast. The counting was made easier by staining the preparation with methyl

violet, but it was still difficult to determine the number of cells which had been only partially disrupted. The Gram staining reaction was finally adopted, this method of differential staining showing both the broken and unbroken cells. Henry and Stacey (1943) reported that unbroken yeast cells give a Gram positive reaction but when the surface is removed from the yeast cell a Gram negative reaction can be demonstrated. An aliquot of the plasmolysed yeast was, therefore, stained with methyl violet, mordanted with iodine and then treated with acid fuschin. Whole cells remaining after solvent treatment were stained blue and the ruptured cells and cell fragments stained red. The per centage of cells broken by treatment with various solvents is shown in Table XIII.

TABLE XIII.

Plasmolysis time (hours)	0.5	1.0	2.0	4.0	6.0
	% of plasmolysed cells				
Toluene	12	41	67	85	95
Benzene	6	18	32	48	60
Ethyl acetate	14	36	71	88	96
Chloroform	8	21	36	51	55

As the enzymic activity of the toluene treated cells, as measured by ATP uptake was greater than that of cells treated with ethyl acetate (Table XIV.), toluene was used as the plasmolysing agent in all further experiments.

TABLE XIV.

Plasmolysing agent	Phosphate uptake (mM.ATP per hour/ml.)	
	4 hr. plasmolysis	6 hr. plasmolysis
Toluene	0.226	0.287
Ethyl acetate	0.214	0.273

The yield of enzyme obtained from the plasmolysed yeast by extraction with 2 volumes of water at 0° for 18 hours, after the method of Berger et al (1946) and Kunitz and MacDonald (1946), was very small. Addition of neutralised sodium sulphide, after the method of Bailey and Webb (1948), gave a higher yield of extracted protein having good hexokinase activity. It was noticed that the addition of sodium sulphide to the aqueous plasmolysed extract raised the pH from about 4.5 to about 6.5. Further experiment showed that the addition of ammonia to the plasmolysed aqueous extract to pH 6.5, gave similar yields of enzyme. The ammonia treatment, requiring 18 hours extraction time at 5° instead of the 48 hours at 20-25° used by Bailey and Webb, was adhered to in all further experiments.

3.4.3. Examination of the previously published Methods for the Preparation of Hexokinase.

From a consideration of the three published methods for the isolation of hexokinase it was seen that the purification required three main operations, (a) inert protein is removed from the yeast protein obtained from the plasmolysed yeast liquor; (b) the hexokinase is obtained in a more concentrated form; (c) after fractionation of this concentrated hexokinase solution with alcohol, crystallisation is accomplished from potassium phosphate solution in the presence of small amounts of ammonium sulphate.

(a) Both Berger et al (1946) and Bailey and Webb (1948) first precipitated all of the protein from the plasmolysed yeast liquor and then removed inert protein, and enzymes, other than hexokinase from the precipitated protein. This was accomplished by Berger et al by acetic acid precipitation of the inert protein, and alcohol fractionation of the remaining protein. Bailey and Webb adsorbed inert material on to calcium phosphate gel, and fractionated out protein other than hexokinase using ammonium sulphate. Kunitz and MacDonald, however, removed the bulk of the inert protein by discarding all of the material which precipitated out on 50 per cent. ammonium sulphate saturation of the original autolysed liquor.

Experiments carried out in this Department (Johnstone, 1953) showed that adsorption onto, and elution from, calcium phosphate gel, gave very variable results, owing to difficulties encountered in taking the adsorbed hexokinase off the gel. This method of purification was therefore, not investigated further.

A method was finally adopted which combined those of Kunitz and MacDonaldand Berger *et al*. The plasmolysed extract was treated with solid ammonium sulphate to 50 per cent saturation and the precipitated protein was discarded. Ammonium sulphate was added to 75% saturation, and inert material was removed from the precipitated protein by means of acetic acid. This procedure gave a three-fold increase of enzyme activity.

3.4.3.

(b) When the protein obtained by treatment (a) was fractionally precipitated with ammonium sulphate, four fractions were obtained having hexokinase activity, the protein precipitating at 65-75% saturation being the most active.

These fractions could be kept at 0° for periods up to four months, without loss of activity, providing about 10 per cent. of ammonium sulphate was present. This concentration of ammonium sulphate is normally present when the protein precipitated by the salt is filtered off, and dissolved in water.

The protein fractions lost hexokinase activity rapidly, even at 0°, when dialysed against water. The ammonium sulphate could be removed by dialysis against 1 per cent. glucose without any loss of enzyme activity, but the dialysed material did not retain hexokinase activity longer than 4 to 5 days at 0°.

The material precipitating between 65 and 75 per cent. saturation was used in most of the phosphorylation experiments (Part II., 2.2.2.2.). As ammonium sulphate-free solutions were necessary for these experiments, dialysis was carried out against 0.5 per cent. glycine solution. Kunitz and MacDonald showed that 1 per cent. glycine protected hexokinase solutions against inactivation but Bailey and Webb stated that glycine did not protect their preparations against spontaneous inactivation. The present investigation showed that dialysis against 0.5 per cent. glycine gave ammonium sulphate-free enzyme solutions which retained their hexokinase activity for periods of from four to seven days, at 0°.

The fraction precipitating between 65 and 75 per cent. saturation was purified further by treatment with alcohol. The protein solution obtained finally had an hexokinase activity 25 times that of the original plasmolysed liquor.

3.4.3.

(c) Attempts were made to crystallise hexokinase from the most active enzyme solution obtained, by the method of Kunitz and MacDonald, without success. The problem of crystallisation was not investigated systematically owing to the small amounts of active material available.

3.4.4. Experimental details of the method evolved.

1. Plasmolysis.

7 lb. of factory fresh yeast (DCL) were crumbled, mixed with toluene (210ml.), and maintained at 38 to 40° for 5½ hours. To the liquid mass were added water (2700ml.) and dilute ammonia solution to pH 6.5-7.0 (about 300ml. of 0.03% v/v ammonia) and the mixture left at 5° for 18 hours. The yeast debris was filtered off on a 25cm. Buchner funnel, through a layer of Hyflo Super Cel on a Whatman No.1 filter paper, giving 3500ml. of clear yellow liquid (called crude extract).

2. Precipitation by ammonium sulphate.

To each 1000ml. of the crude extract was added solid ammonium sulphate (314g.) to give 50% saturation, the precipitated protein was filtered off at 8-12°, and discarded. The clear filtrate was brought to 75% saturation by addition of a further 173g. of ammonium sulphate per litre. The precipitate was allowed to settle at 5° for 18 hours, was

filtered off, and dissolved in 4 volumes of cold water. (Fraction I.).

3. Dialysis.

Fraction I. was dialysed against 1% glucose solution for 24 hours at 0° with 5 changes of glucose solution. The ammonium sulphate concentration was zero after this time. (Fraction II.).

4. Acid precipitation.

The pH of Fraction II. was adjusted to 4.7, at 0°, by the slow addition of M acetic acid (about 25ml.). The precipitate was filtered off in the cold through Hyflo Super Cell on Whatman No.1 paper. The pH of the filtrate was adjusted, at 0°, to 5.6 by addition of N-sodium hydroxide (about 15ml.). (Fraction III.).

5. Ammonium sulphate fractionation.

Fraction III. was brought to 55% saturation by addition of solid ammonium sulphate (182.0g.), the precipitate was filtered off and dissolved in 2 volumes of cold water, (Fraction IV.). The saturation was increased to 65% by addition of ammonium sulphate (41g.) to give Fraction V. The saturation was increased to 75%, and then 85%, by addition of 43.0g. and 44.5g. ammonium sulphate respectively, giving Fractions VI. and VII.

6. Fractionation with ethanol.

Fraction VI. was dialysed against 1% glucose at 0° for 20 hours, with three changes of glucose. Absolute alcohol at -10° was added to the cooled solution (62ml.) to give a final concentration of 35% (v/v). The precipitate was filtered off under gavity at 0° and discarded and the ethanol content of the filtrate raised to 60% (v/v). The precipitate was quickly spun down in a cooled centrifuge and dispersed in 10% ammonium sulphate solution (4ml.).

TABLE XV.

Summarisation of Purification Data

Stage	Volume ml.	Activity (Hexokinase units/ml.)	Total Activity (Hexokinase units)
1. Plasmolysis liquor Crude extract.	3,500	12	42,000
2. 50-75% precipitate. (Fraction I.)	250	81	20,250
3. Dialysis. (Fraction II.)	480	148 36	71,280
4. Acid precipitation (Fraction III.)	520	30	15,600
5. 0-55% precipitate (Fraction IV.)	20	42	840
55-65% precipitate (Fraction V.	25	43	1,150
65-75% precipitate (Fraction VI.	48	206	9,900
75-85% precipitate (Fraction VII.)	18	111	2,000
6. Alcohol precipitation	4	308	1,224

The enzyme preparation used in the glucosone experiments, (Part II. 2.2.2.1.) had an activity of approximately 90 hexokinase units per mɸl. It was obtained by fractionating the 50-75% precipitate (Fraction I. Table XV.) with ammonium sulphate. The fraction precipitating between 65 and 75% saturation was dissolved in water, and dialysed against 1% glycine or distilled water.

The amount of ammonium sulphate necessary to give a required saturation was calculated from the equation given by Kunitz (1952).

Amount of ammonium sulphate required

$$= \frac{53.3\ (S_2 - S_1)}{1 - 0.3 \times S_2}$$

where S_1 = initial ammonium sulphate concentration,

and S_2 = final ammonium sulphate concentration.

Dixon (1953) has prepared a nomograph from which the amount of ammonium sulphate for a required saturation can be calculated.

The unit of activity reported in this investigation is that amount of enzyme which causes a carbon dioxide output of 1cub.mm. per minute under the conditions of the test system. An amount of protein solution, suitably diluted, equivalent to 20 units of hexokinase, was contained in each flask in the experiments described in Part II. 222.

4. Analytical Methods.

4.1. Barium.

Barium was estimated gravimetrically as barium sulphate.

4.2. Nitrogen.

Nitrogen was estimated using the Kjeldahl method.

4.3. Phosphorus.

Inorganic phosphorus was determined by the method of Fiske and SubbaRow (1925), and by the method of King (1932). The relative densities were measured with an E.E.L. colorimeter using an Ilford 208 filter.

Total phosphorus: The material was digested with 10N-sulphuric acid and hydrogen peroxide as described by LePage (1949), or with perchloric acid and hydrogen peroxide as described by King (1932), and the resulting inorganic phosphate determined by either of the methods reported above.

4.4. Reducing Sugars.

Reducing sugars were determined by the method of Nelson (1944) using the modified reagents of Somogyi (1945, 1952). It was found that ammonium sulphate at concentrations greater than 2%, interfered with the sensitivity of the method.

The E.E.L. colorimeter was used to measure relative densities, using an Ilford 608 filter.

4.5. DPN.

DPN was assayed by measuring the optical density of a solution of the reduced material at 340mμ, using a Beckmann spectrophotometer. The method followed was that of LePage (1947).

5. Chromatographic Techiques.

Chromatographic analyses were carried out to check the homogeneity of the carbohydrates used in these studies and also to determine the nature of the phosphorylated derivatives produced enzymatically or chemically. The studies reported here are divided into three parts, (I.) the separation of carbohydrates, (II.) the separation of sugar phosphates, and (III.) chromatography of the products of the hexokinase reqction.

5.1. Separation of Carbohydrates.

D-Glucosone, D-glucose and D-fructose were separated in phenol-water (80:20) containing 0.5% benzoinoxime (Plate 5). A chromatogram was irrigated at room temperature for 18-22 hours, dried at 60°, and then washed with ether. The dried chromatogram was passed through a 0.5% chloroform solution of triphenyltetrazolium chloride (TTC) (Trevelyan, Procter, and Harrison, 1950), dried at 100° for 2 minutes, and passed through a 0.1M alcoholic solution of caustic soda. After being dried at 60° for a few minutes the chromatogram was heated at 100° for 5-10 minutes. The sugars appeared as bright red spots on a white ground, and excess reagent was removed by washing with warm Teepol-water solution. This method has proved to be highly satisfactory with all of the reducing substances tested, but some comments on the procedure and the materials used are appended.

PLATE 5.

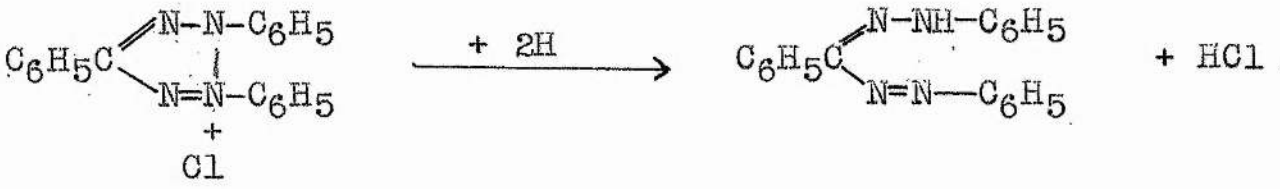
PLATE 5.

1. Fructose.
2. Glucosone.
3. Glucose.
4. Glucosone (a), glucose (b), fructose (c).

The chromatogram was run on Whatman No. 2 paper, in phenol-water, for 22 hours at 16°.

It was found that the presence of excess phenol on the chromatogram caused much background colour after the final heating at 100°. Air drying for long periods removes all traces of phenol but it found that ether washing removed sufficient phenol from the paper to prevent extraneous colour formation. The drying at 100° after treatment with TTC is not absolutely necessary, but deeper final colours are obtained in this manner. Excess heating at this stage, however, again produces much unwanted discoloration. Experiment showed that the final colour production could be controlled very accurately by means of the sodium hydroxide concentration of the alkali bath. At a sodium hydroxide concentration of 0.25N the sugar spots appeared within one minute on heating at 100°, the chromatogram becoming completely red in about three minutes. Continued dilution of the alcoholic sodium hydroxide with ethanol, down to 0.1N or 0.05N allowed more latitude in heating time, about 6-7 minutes heating at 100° producing bright red spots on a white ground. The method of Wallenfels (1950) using 2% TTC in 1.0N sodium hydroxide gave good results also, but the control of the colour production is not as definite as that of the method used.

Triphenyltetrazolium chloride forms a red formazan on reduction, according to the equation:



It has been known for many years that the substance is reduced by plant tissues, reduction by a dehydrogenase being the probable cause of the effect. TTC gives the characteristic reaction with plant tissues at pH 7.2 but reducing sugars will not reduce the substance below pH 11.0, and glutathione, cysteine or ascorbic acid not below pH 9. It has been found, however, that glucosone will reduce TTC at pH 8.0. This feature is of some value as chromatograms were irrigated, in some cases, with solvents which did not give much separation of glucose and glucosone. In such cases the chromatogram was dipped, after TTC treatment, into a bath of potassium acetate in alcohol. Consequent heating at 100° produced the formazan only where glucosone was sited, the glucose spot appearing only after treatment with alcoholic alkali and further heating at 100°. As glucosone also produces the formazan at a lower temperature than does glucose, using the alcoholic-alkali bath, identification of the osone in the presence of glucose can be accomplished by careful control of the final heating.

A large number of solvents were tested in an endeavour to obtain maximum separation of the carbohydrates used. Neither s-collodine-water nor n-butanol-acetic acid-

PLATE 6.

Solvent front

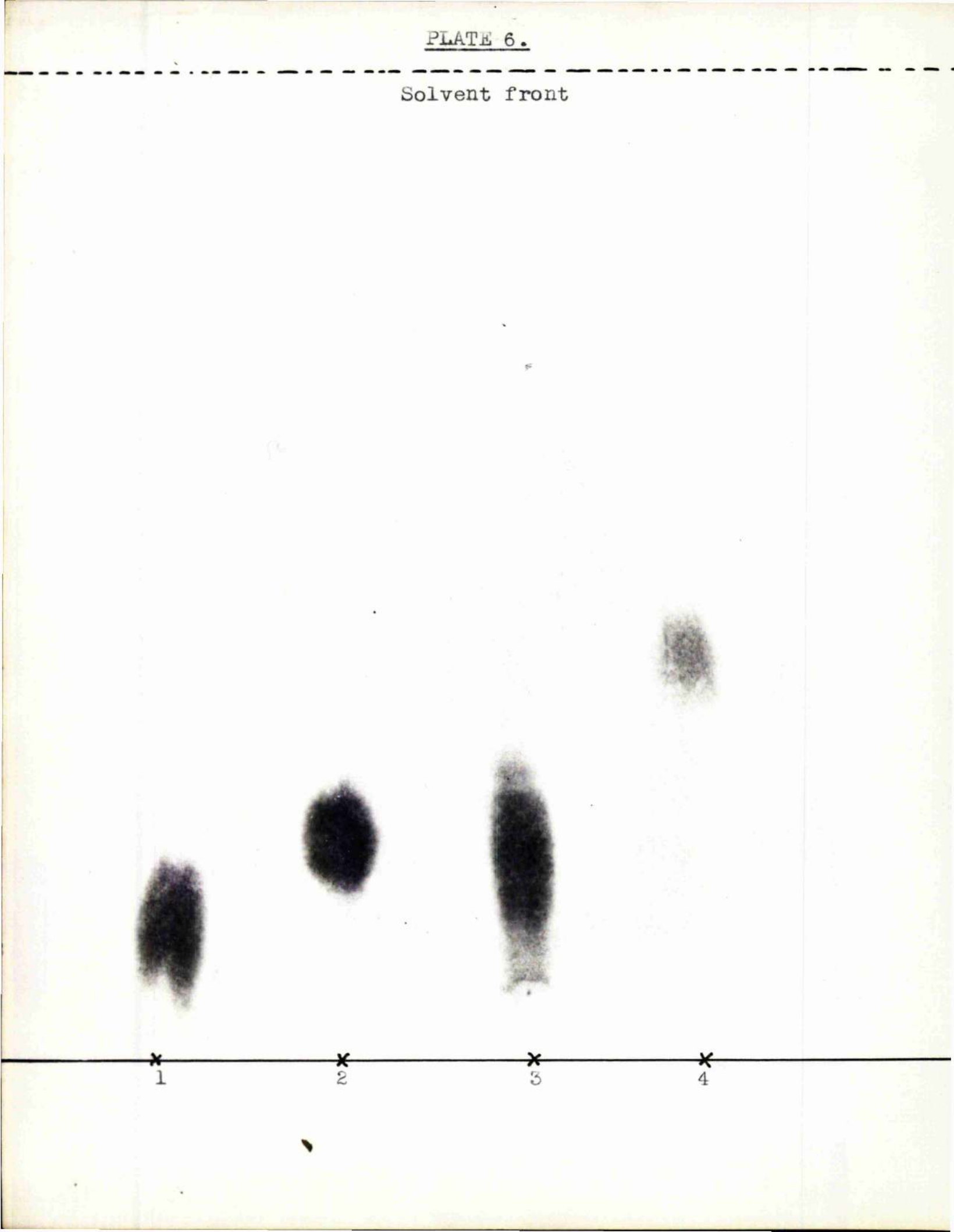


PLATE 6.

1. Glucose.
2. Fructose.
3. Glucosone.
4. 2-deoxy-glucose.

The chromatogram was run on Whatman No.2 paper, in butanol-pyridine-water (5:3:3), for five hours at 37°.

water (Partridge, 1948) gave as good a separation of glucose and glucosone as did phenol-water. The glucosone gave some tailing in butanol-acetic acid, and Fewster (1953) has shown that glucosone can be resolved into three components using this system, when the carbohydrate is chromatographed immediatel after being taken into solution. An equilibrium solution is produced after a few hours in aqueous solution which shows only one definite spot with some tailing. It was found in the present experiments, that the glucosone equilibrium mixture can be resolved into one main spot plus a second spot having a higher R_f, in phenol-water.

The separation of glucosone from glucose was found to be very poor in the three phase systems containing components such as ethylacetate, pyridine and water or ethylacetate acetic acid and water (Jermyn and Isherwood, 1949). The solvent mixtures, n-butanol-ethanol-water, and n-butanol-pyridine-water suggested by Hough, Jones and Wadman (1950) did give fairly good separation of the sugars. The second of these two solvents was found to be more suitable for the present work and various combinations of the constituents were tested. It was found that a mixture of n-butanol-pyridine-water (5:3:3) run at 37° gave some separation of glucose, glucosone and fructose on running the chromatogram for 20cms. (Plate 6). Glucosone could not be identified on chromatograms developed with solvents containing ammonia

on account of the lability of the osone in these media.

5.2. Separation of Sugar Phosphates.

In order to identify the enzymatically formed phosphorylated sugars, a study was made of the methods for separating the hexose phosphates on paper chromatograms.

The method of Hanes and Isherwood (1949) was found to be suitable for the separation of glucose-1-phosphate, fructose-1-phosphate, glucose-6-phosphate and fructose-1:6-di-phosphate, using ethyl-acetate-pyridine-water (100:45:100) run on Whatman No.54 paper at room temperature for 18 hours. Phosphates were detected by spraying the paper with the per-chloric acid-HCl-molybdate solution described by Hanes and Isherwood (1949). After heating at 100° for 5-10 minutes the chromatogram was sprayed with a reducing solution of amidol (2.0g.), sodium bisulphite (5.0g.), water (35ml.) and acetone (15ml.), when the phosphates appeared as blue spots on a white or pale blue ground. Much background colour was obtained using either Whatman No.54 paper or Whatman No.1 paper washed with 2N-acetic acid and water.

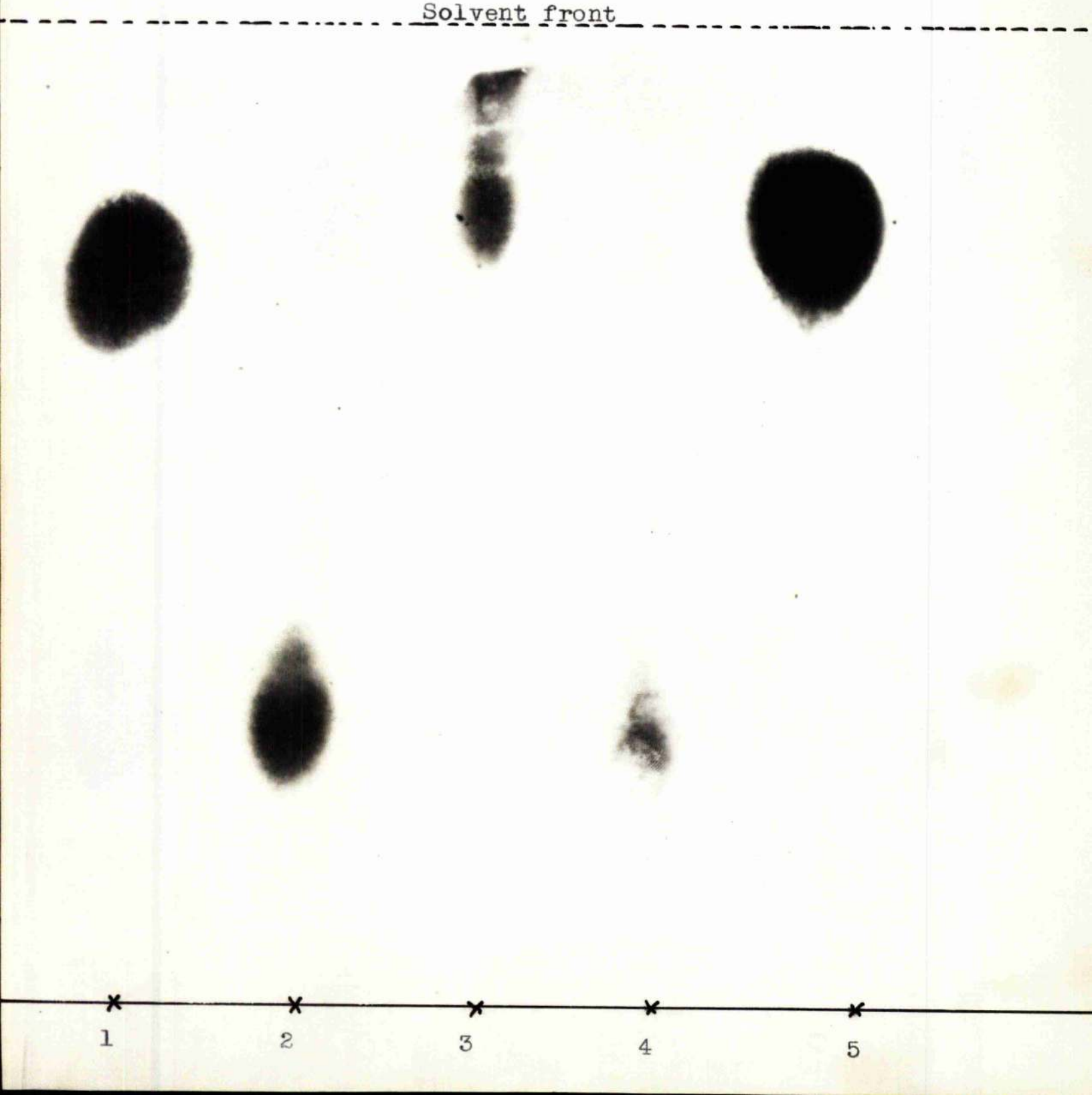
The phosphates were also detected by the method of Wade and Morgan (1953) using ferric chloride and sulphosalicylic acid. Good results were obtained only when 10% sulphosalicylic acid was used after irrigation with non-acidic solvents.

Some separation of the phosphates was also obtained using the methyl cellosolve-methyl ethyl ketone-N-ammonia (3:2:1) solvent of Mortimer (1952). The separation was found to be much better using downward development at 18°, when the solvent was run to the bottom of the paper, dried off, and then run down again. Any further repetition of the procedure gave a greater separation of the hexose phosphates, but the final spots became very diffuse.

Rather better separation of the sugar phosphates was obtained by developing the chromatograms in the cold, in the methyl alcohol-formic acid-water (80:15:5) solvent of Bandurski and Axelrod (1951), or acetone-35% formic acid (60:40) as suggested by Burrows, Grylls and Harrison (1953). The chromatograms were run on Whatman No.1 paper (acid washed) in a 29 x 10cms. jar in the refrigerator at 5°. Using a 28 x 23cms. paper it was possible to place 5 substances along the shorter side of the paper, on a line drawn 4cms. from the edge. The paper was then folded six times so that each spot moved up a strip 24cms. long by 5cms. wide. The chromatogram was developed, without any equilibration,

by dropping the folded paper into the jar containing about 0.5cms. of solvent. Both of the solvent mixtures used travelled 20-24cms. in 5½-6 hours.

PLATE 7.



5.3. Chromatographic Analysis of the Products of the Hexokinase Reaction.

As a further check on the phosphorylative ability of the hexokinase preparation used in the specificity experiments, the contents of the Warburg flasks were, in many cases, chromatographed after activity measurements. A solvent system was evolved which would separate phosphate esters from the parent carbohydrate, and thus allow of their identification on the same chromatogram.

A large number of solvents were tested using various proportions of the substituents of the three phase systems such as, ethyl acetate-acetic acid-water; ethyl acetate-pyridine-water; butanol-ethanol-water; butanol-acetic acid-water; amyl alcohol-formic acid-water; amyl alcohol-ethyl acetate-formamide; ethyl acetate-pyridine-formamide. Test chromatograms were run at 40° with Whatman No.1 and No.2 papers, using glucose, glucosone, fructose and glucose-6-phosphate. The development was carried out in a 29 x 10cms. jar in an incubator, and the test substances were identified using TTC in the manner previously described.

It was found that the mixtures containing n-butanol did not produce much movement of the sugar phosphate, and replacement of the n-butanol by amyl alcohol gave higher phosphate R_f values but did not separate them sufficiently from the hexoses. The addition of benzene to butanol-acetic

PLATE 7.

1. Glucose.
2. Glucose-6-phosphate.
3. Glucosone.
4. Fructose-1-phosphate.
5. Fructose.

The chromatogram was run on Whatman No.2 paper, in amyl alcohol-ethyl acetate-formamide (4:2:5), for 5½ hours at 37°.

acid-water gave much higher sugar R_f values, by decreasing the water content of the mixture. The solvent layer of an n-butanol-acetic acid-benzene-water (5:1:3:2) mixture, for example, gave good separation of the phosphate and sugars, and it also found that this system could be used to separate mono-and disaccharides.

Experiments with different concentrations of ethyl acetate, formamide and pyridine showed that pyridine reduced the R_f values of sugars developed with this mixture. The replacement of the pyridine by various alcohols, however, increased the sugar R_f but decreased that of the phosphate. Amyl alcohol was found to give the best separation of the phosphorylated sugar and the hexoses under the test conditions. As the movement of the phosphates on the chromatogram was controlled mainly by the amount of formamide present, various mixtures of ethyl acetate-amyl alcohol-formamide were tested until the mixture best suited for the present investigation, was found. This mixture (ethyl acetate-amyl alcohol-formamide, 1:2:3) was used thereafter in most chromatographic runs of this type. Glucose-6-phosphate and fructose-1-phosphate were not separated by this solvent mixture, or by different proportions of these solvents, however. (Plate 7.).

The contents of the Warburg flasks were prepared for chromatography by first precipitating the protein by addition of 0.25 volumes of 10% trichloroacetic acid. The

protein-free filtrate was neutralised with ammonia or sodium hydrogen carbonate, and the cations removed with Amberlite I.R. 120 ion exchange resin. The deionised solution was placed, without further concentration, onto a Whatman No.2 paper, 10-15 µl. of the solution being applied. The chromatogram was developed for 4½ - 5 hours at 40°, without previous equilibration, reproducible results being obtained providing the solvent was renewed after two runs.

SUMMARY

1. The inhibitory effect of glucosone on yeast fermentation was shown to be specific for the D-isomer, and exerted at some point before the breakdown of fructose-1:6-diphosphate.

2. The results of the fermentation experiments are analysed kinetically, and glucosone shown to be a pseudo-irreversible inhibitor, of the type described by Ackermann and Potter (1949). It is shown that the effects produced by glucosone in animals can be correlated with this finding.

3. The osone is shown to be phosphorylated by ATP in the presence of hexokinase.

4. It is suggested that the inhibitory effect produced by glucosone on yeast fermentation, is due to the slow dissociation of a glucosone phosphate from the hexokinase molecule.

5. The effect of hexokinase on a number of glucose analogues is reported, and indications are given of the high degree of specificity exhibited by the enzyme.

6. Methods are described for the preparation of an actively fermenting acetone-dried extract, and a maceration juice, from bakers' yeast. The preparation and some properties of cold-treated bakers' yeast are also described.

7. A method is described for the separation of the substrates and products of the hexokinase reaction, on paper chromatograms.

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APPENDIX

Tables 1 to 38.

TABLE 1. Data for Fig.I.

D-Glucose	0.025M.			
Yeast mg. wet wt./3ml.	5	10	25	40
Time min.	μl CO_2			
5	10	19	50	69
10	32	59	142	228
15	52	101	234	387
20	69	145	332	
25	92	187	420	
30	111	231		
35	131	275		
40	150	316		

TABLE 2. Data for Fig.II.

D-Glucose	0.025M.				
Yeast	25mg. wet wt./3ml.				
KCN	2.6×10^{-4}M.				
D-Glucosone (M)	-	0.050	0.100	0.150	0.175
Time min.			μl CO_2		
5	67	34	28	14	7
10	133	95	70	45	15
15	226	157	112	73	25
20	318	214	150	98	33
25	410	278	196	124	40
30		340	238	150	49
35		396	277	175	54
40			315	197	57

TABLE 3. Data for Table I.

Yeast	25mg. wet wt./3ml.							
D-Glucose (M)	0.010	0.010	0.025	0.025	0.040	0.040	0.050	0.050
D-Glucosone (M)	-	0.040	-	0.100	-	0.160	-	0.200
Time min.	μl CO_2							
5	38	20	50	25	51	37	60	40
10	112	56	135	70	155	85	180	96
15	190	95	228	110	261	133	300	148
20	260	133	316	153	368	174	414	203
25	335	176	412	200	470	220	-	260
30	413	220	-	242	-	265	-	318

TABLE 4. Data for Fig.III.

Yeast	25mg. wet wt./3ml.					
D-Glucose (M)	0.025	0.010	0.015	0.020	0.025	0.030
D-Glucosone (M)	-	0.10	0.10	0.10	0.10	0.10
Time min.	μl CO_2					
5	57	12	12	26	35	40
10	135	15	20	55	77	95
15	223	18	30	79	120	151
20	309	21	37	103	165	210
25	390	24	45	125	207	265
30	-	27	50	148	252	317
35	-	28	55	170	295	370
40	-	29	58	185	325	-

TABLE 5. Data for Fig.IV. and Table II.

Yeast	10mg. wet wt./3ml.				
D-Glucose (M)	0.01	0.01	0.01	0.01	0.01
D-Glucosone (M)	-	0.010	0.025	0.050	0.075
Time min.			μl CO_2		
5	18	12	8	8	6
10	42	30	16	12	8
15	67	56	24	16	12
20	92	71	39	24	20
25	118	93	51	32	25
30	143	111	64	40	31
35	168	130	76	47	36
40	196	149	89	55	40
45	223	167	98	59	42
50	250	183	101	61	44

TABLE 6. Data for Table II.

Yeast	10mg. wet wt./3ml.				
KCN	2.6×10^{-4}M.				
D-Glucose (M)	0.01	0.01	0.01	0.01	0.01
D-Glucosone (M)	-	0.010	0.025	0.050	0.075
Time min.	μl. CO_2				
5	19	12	10	6	4
10	40	34	23	19	12
15	62	49	34	24	16
20	88	68	46	31	18
25	111	85	55	36	20
30	134	104	64	41	21
35	160	120	70	43	21
40	182	136	78	45	22
45	206	153	86	46	23
50	226	191	94	48	25

TABLE 7. Data for Fig.VI.

Yeast, wet wt.	10mg./3ml.																
D-Glucose (M)	0.005				0.0075			0.010						0.020			
Time min.	μl CO_2																
5	14	10	10	11	13	16	16	22	18	19	21	21	19	28	28	33	
10	30	24	24	27	30	35	35	50	44	46	45	45	40	60	64	66	
15	50	42	41	45	54	56	60	78	70	70	73	70	64	88	93	98	
20	69	60	59	63	76	78	84	105	94	96	99	94	90	121	128	133	
25	87	79	78	81	103	102	108	130	120	125	125	118	115	154	162	168	
30	106	98	93	104	127	124	132	153	146	150	151	144	140	186	198	202	
35	127	118	112	123	152	146	157	176	170	175	176	168	164	198	230	235	
40	148	138	130	146	174	167	180	204	192	203	200	192	190	250	260	268	
45	164	160	150	161	198	188	204	228	216	230	227	218	216	270	290	300	
50	184	184	168	178	220	208	228	252	240	255	250	241	240	310	318	328	
Output per 10-40 min.	118	114	106	119	144	132	145	154	148	157	155	147	150	190	196	202	
Average	114				141			152						196			
Rate per hour	228				282			304						392			

TABLE 8. Data for Fig.VI.

Yeast	10mg. wet wt./3ml.														
D-Glucosone	0.01M.														
D-Glucose (M.)	0.005				0.0075				0.010				0.020		
Time min.	μl CO_2														
5	10	8	6	8	12	14	14		12	11	10	12	20	22	22
10	20	20	18	18	27	31	32		27	25	29	26	42	46	49
15	31	30	30	29	42	45	46		49	45	50	46	64	72	74
20	45	43	44	44	58	62	62		70	66	71	66	88	96	102
25	56	52	56	54	75	80	80		90	86	92	87	112	121	128
30	66	64	67	63	91	96	96		110	106	111	107	138	148	153
35	79	76	70	74	106	110	113		129	125	129	127	165	171	178
40	90	87	94	86	124	130	126		148	145	147	147	193	194	198
45	102	97	106	96	140	145	138		166	164	164	166	217	215	215
50	114	106	118	107	154	161	150		179	181	180	182	240	231	225
Output per 10-40 min.	70	67	76	68	97	99	94		121	120	118	121	151	148	151
Average	70				97				120				150		
Rate per hour	140				194				240				300		

TABLE 9. Data for Fig.VI.

Yeast	10mg. wet wt./3ml.															
D-Glucosone	0.025M.															
D-Glucose (M)	0.005				0.0075			0.010					0.020			
Time min.	μl CO_2															
5	64	66	6	6	12	12	13	6	6	8	10	9	16	16	17	
10	11	8	12	13	22	23	22	16	18	22	26	20	32	34	36	
15	19	16	20	22	32	36	36	30	31	34	38	33	50	54	54	
20	26	23	28	30	43	48	48	44	46	48	50	46	66	71	70	
25	33	30	34	35	55	58	60	56	60	62	62	59	84	89	86	
30	40	36	39	44	66	68	70	70	73	76	80	72	100	106	103	
35	47	43	47	51	75	78	79	82	86	90	92	84	116	123	120	
40	54	52	54	58	83	85	88	94	99	104	103	96	134	140	138	
45	61	60	60	66	91	93	96	106	112	120	115	108	150	153	154	
50	68	67	66	72	98	101	104	120	125	134	127	121	165	164	168	
Output per 10-40 min.	43	44	42	45	61	62	66	78	81	82	77	76	102	106	102	
Average	43				63			79					104			
Rate per hour	86				126			158					208			

TABLE 10. Data for Fig.VI.

Yeast	10mg. wet wt./3ml.											
D-Glucosone	0.050M.											
D-Glucose (M.)	0.0075				0.010					0.020		
Time min.	μl CO_2											
5	4	8	10	9	4	6	6	7	8	10	11	12
10	10	14	17	17	10	12	14	14	16	24	25	28
15	18	21	25	26	18	22	24	22	24	36	40	41
20	26	27	33	32	26	31	35	29	32	50	54	56
25	38	33	40	39	34	40	44	36	39	64	68	70
30	40	38	46	45	42	47	50	44	44	78	82	85
35	46	43	52	50	51	54	58	51	51	94	96	100
40	51	49	58	55	60	62	65	60	56	108	110	115
45	56	54	64	59	68	67	71	66	62	122	122	128
50	60	59	70	62	73	70	78	74	68	136	134	140
Output per 10-40 min.	41	35	41	38	50	50	51	46	40	84	85	87
Average	39				48					85		
Rate per hour	78				96					170		

TABLE 11. Data for Fig.VI.

Yeast	10mg. wet wt./3ml.							
D-Glucosone	0.075M.							
D-Glucose (M.)	0.010					0.020		
Time min.	μl CO_2							
5	6	6	7	5	5	9	10	12
10	13	10	15	8	10	19	21	24
15	20	16	21	14	16	30	32	35
20	25	21	25	20	24	39	41	45
25	30	26	29	24	30	38	51	55
30	36	30	33	30	36	48	60	65
35	41	34	37	36	42	66	69	74
40	46	38	41	42	49	74	78	83
45	50	41	42	48	54	82	86	92
50	54	45	44	54	59	89	94	100
Output per 10-40 min.	33	28	26	34	39	55	57	59
Average			32				57	
Rate per hour			64				114	

TABLE 12. Experimental points for Fig.VI.

calculated from Tables 7-11.

D-Glucosone (M)		-		0.010		0.025		0.050		0.075	
D-Glucose (M) S.	$\frac{1}{S}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$
0.0050	200	22.8	0.0438	14.0	0.0715	8.4	0.1190	-		-	
0.0075	136	28.2	0.0355	19.6	0.0510	12.6	0.0794	7.8	0.1280	-	
0.0100	100	30.4	0.0329	24.0	0.0416	15.8	0.0632	9.6	0.1040	6.4	0.1560
0.0200	50	39.2	0.0255	30.0	0.0330	20.8	0.0480	17.0	0.0580	11.4	0.0860

v = μl CO_2 per hour per mg. wet wt. of yeast.

TABLE 13. Theoretical points for Fig.VI.

D-Glucosone (M)	$\sum\left(\frac{1}{v}\right)$	$\sum\left(\frac{1}{S}\right)$	$\sum\left(\frac{1}{S}\right)$	$\sum\left(\frac{1}{v} \times \frac{1}{S}\right)$	$\frac{1}{v}$ at D-Glucose concentration (M) 0	.005	.0075	.010	.020
-	.1377	486	70996	18.15	.020	.044	.036	.032	.026
.010	.1971	486	70996	27.05	.016	.067	.050	.042	.029
.025	.3090	486	70996	43.20	.019	.115	.074	.067	.043
.050	.2900	286	30996	30.70	.019	-	.131	.101	.060
.075	.2420	150	12500	19.90	.019	-	-	.156	.086

The points are calculated by the method of least squares from data on Table 12, using the equations:-

$$\sum\left(\frac{1}{v}\right) - a_o n - a \sum\left(\frac{1}{S}\right) = 0$$

$$\sum\left(\frac{1}{v}\right) \times \frac{1}{S} - a_o \sum\left(\frac{1}{S}\right) - a \sum\left(\frac{1}{S}\right)^2 = 0$$

where $$\sum\left(\frac{1}{v}\right) = a_o + a \sum\left(\frac{1}{S}\right)$$

TABLE 14. Data for Fig.V.

Yeast	10.0mg. wet wt./3ml.				
D-Glucose	0.01M.				
D-Glucosone (M)	-	0.025	0.010	-	-
L-Glucosone (M)	-	-	-	0.025	0.010
Time min.	μl CO_2				
5	19	6	7	22	22
10	46	18	10	47	44
15	70	31	12	71	69
20	96	46	13	98	95
25	125	60	13	126	122
30	150	73	14	154	148
35	175	86	16	177	175
40	203	99	18	205	201
45	230	112	20	232	227
50	255	125	21	259	253

TABLE 15. Data for Fig.VII.

Yeast wet wt./3ml.	5mg.				10mg.				15mg.				25mg.			
D-Glucose (M)	0.01		0.01		0.01		0.01		0.01		0.01		0.01		0.01	
D-Glucosone (M)	-		0.03		-		0.03		-		0.03		-		0.03	
Time min.	μl CO_2															
5	8	10	9	7	20	19	11	13	18	23	20	24	30	35	38	40
10	20	21	14	12	43	40	22	26	50	50	42	47	86	100	71	80
15	30	34	20	17	68	64	32	39	86	82	60	66	175	190	106	111
20	42	44	23	21	94	90	43	51	102	106	78	85	280	292	140	145
25	54	56	28	25	120	115	56	63	160	150	98	104	-	-	172	178
30	66	67	32	30	145	140	67	74	198	190	118	123	-	-	204	216
35	78	80	36	33	170	166	78	84	238	226	136	141	-	-	218	245
40	92	93	41	42	195	190	88	93	276	260	152	156	-	-	236	276
45	106	106	43	48	220	216	98	102	314	300	166	170	-	-	290	303
50	121	118	43	48	246	240	107	110	356	336	181	182	-	-	314	328
Output per 10-40 min.	72	72	25	27	152	150	66	67	226	216	110	109			165	196
Rate per hour	144		52		302		132		436		220		772		360	

TABLE 16. Data for Fig.VIII.

Yeast	10mg. wet wt./3ml.							
Pre-incubation time (min.)	zero	zero	10	20	30	40	50	120
D-Glucose (M)	.01	.01	.01	.01	.01	.01	.01	.01
D-Glucosone (M)	-	.025	.025	.025	.025	.025	.025	.025
Time min.	μl CO_2							
5	22	8	10	6	6	5	5	6
10	50	21	16	9	8	9	7	8
15	78	36	24	13	11	11	10	10
20	105	49	31	17	14	13	12	11
25	130	64	36	21	17	16	13	12
30	153	71	41	24	20	17	13	13
35	176	81	46	27	22	18	14	13
40	204	94	52	30	23	19	15	14
45	228	105	57	34	24	20	15	14
50	252	113	62	36	25	20	16	14
Rate per hour	300	146	72	42	30	20	16	14
% Inhibition		49	24	13.8	10	6.6	5.5	4

TABLE 17. Data for Fig.IX.

Yeast	10mg. wet wt./3ml.			
	Flask A	A1	Flask B	B1
D-Glucose (M)	0.01	-	0.01	0.01
D-Glucosone (M)	0.04	0.04	0.04	-
Time min.	μl CO_2			
0	tip glucosone		tip glucose	
5	5	4	21	20
10	6	6	50	53
15	Add glucose → 7	8	Add glucosone → 75	77
20	10	9	102	104
25	14	9	122	132
30	17	10	138	157
35	21	10	153	182
40	26	11	168	207
45	32	11	180	232
50	36	11	194	256
55	43	12	206	282
60	48	13	214	309
65	54	13	225	334

TABLE 18. Data for Fig.X.

Yeast	10mg. wet wt./3ml.					
D-Glucose (M)	.0075		.01		.02	
D-Glucosone (M)	.025		.025		.025	
Time min.	μl CO_2					
5	5	4	9	9	8	8
10	7	5	16	15	16	18
15	10	9	21	20	25	26
20	14	10	26	25	33	34
25	17	18	32	30	40	42
30	20	22	37	35	47	49
35	23	26	42	39	54	56
40	26	30	47	43	60	63
45	29	33	50	47	66	70
50	30	36	55	52	75	76
Output per 10-40 min.	19	25	31	28	44	45
Rate per hour	44		59		89	

TABLE 19. Data for Fig.X.

D-Glucosone (M)		- -		0.025	
D-Glucose (M) S.	$\frac{1}{S}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$
0.0075	136	28.2	.0355	4.4	.227
0.0100	100	30.4	.0329	5.9	.169
0.0200	50	39.2	.0255	8.9	.112

D-Glucosone (M)	$\sum\left(\frac{1}{S}\right)$	$\sum\left(\frac{1}{S}\right)$	$\sum\left(\frac{1}{v} \times \frac{1}{S}\right)$	$\frac{1}{v}$ at glucose concentration 0	.0075	.01	.02
-	286	30996	9.38	.020	.036	.032	.026
.025	286	30996	53.47	.032	.222	.172	.104

TABLE 20. Data for Fig.XI. and XII.

Yeast	25mg. wet wt./3ml.							
D-Glucose (M)	0.005			0.010			0.020	
Time min.	μl CO_2							
5	24	25	20	25	36	30	35	30
10	51	52	50	55	70	65	71	65
15	78	80	80	94	104	100	107	105
20	105	106	111	140	137	135	144	152
25	131	134	142	182	170	168	180	197
30	157	160	170	222	204	203	219	243
Output per 0-20 min.	108			137			148	
Rate per hour	324			411			444	

TABLE 21. Data for Fig.XI. and XII.

Yeast	25mg. wet wt./3ml.					
D-Glucosone	0.01 M.					
D-Glucose (M)	0.005		0.010		0.020	
Time min.	μl CO_2					
5	17	18	19	13	30	27
10	33	34	38	32	62	57
15	48	48	58	53	96	84
20	60	58	78	75	126	110
25	69	66	96	94	154	144
30	76	73	113	113	178	174
Output per 0-20 min.	59		76.5		118	
Rate per hour	177		229.5		354	

TABLE 22. Data for Fig.XI. and XII.

Yeast	25mg. wet wt./3ml.					
D-Glucosone	0.02 M.					
D-Glucose (M)	0.005		0.010		0.020	
Time min.	μl CO_2					
5	12	11	12	15	20	25
10	20	23	25	29	42	48
15	28	35	38	43	61	71
20	35	45	51	58	82	93
25	41	54	62	72	99	115
30	45	61	72	85	114	131
Output per 0-20 min.	40		54.5		87	
Rate per hour	120		163.5		261	

TABLE 23. Data for Fig.XII.

D-Glucosone (M)	$\frac{1}{S}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$	v	$\frac{1}{v}$
		-		.01		.02	
D-Glucose (M)							
.005	200	13.0	.077	7.05	.144	4.80	.208
.010	100	16.5	.060	9.15	.109	6.55	.153
.020	50	17.6	.057	14.1	.071	10.40	.096

D-Glucosone (M)	$\sum\left(\frac{1}{v}\right)$	$\sum\left(\frac{1}{S}\right)$	$\sum\left(\frac{1}{S}\right)^2$	$\sum\left(\frac{1}{v} \times \frac{1}{S}\right)$	$\frac{1}{v}$ at substrate concentration			
					0	.005	.01	.02
-	.194	350	52500	24.25	.048	.076	.062	.055
.01	.324	350	52500	43.25	.054	.152	.104	.079
.02	.457	350	52500	61.7	.058	.211	.136	.095

TABLE 24. Data for Table III.

Yeast	25mg. wet wt./3ml.					
D-Glucose	0.01 M.					
ATP (M)	-		0.001		0.006	
D-Glucosone (M)	-	0.015	-	0.015	-	0.015
Time min.	μl CO_2					
5	36	14	30	16	30	19
10	70	29	70	34	74	38
15	103	33	111	51	122	53
20	135	57	151	69	173	69
25	168	69	193	85	223	84
30	202	80	236	100	274	99
Output per 0-20 min.	135	57	151	69	173	69
Rate per hour	405	171	453	207	519	207

TABLE 25. Data for Fig.XIII.

Yeast extract	1.0ml.				
Fructose diphosphate (M)	0.01	-	0.01	0.01	0.01
D-Glucosone (M)	-	0.08	0.02	0.04	0.08
Time min.			μl CO_2		
5	3	3	4	7	5
10	14	5	16	18	11
15	29	6	31	34	24
20	46	6	50	52	28
25	65	7	69	70	54
30	84	8	88	90	70
35	104	9	106	108	90
40	123	11	123	130	109
45	142	11	140	148	128

TABLE 26. Data for Table IV.

Yeast extract	1.0ml.					
D-Glucose	0.01M.					
D-Glucosone (M)	-	.005	.010	.015	.020	.030
Time min.	μl CO_2					
5	8	7	6	6	5	3
10	19	16	12	13	8	4
15	36	26	20	18	10	5
20	52	38	29	24	12	6
25	68	50	38	28	14	6
30	85	65	48	32	16	7
35	103	79	57	35	18	7
40	121	90	67	38	20	7
45	137	99	74	41	21	7
50	153	107	82	43	23	7
Output per 10-40 min.	102	84	55	25	12	3
Rate per hour	204	148	110	50	24	6

TABLE 27. Data for Table V.

Yeast extract	1.0ml.					
D-Glucose	0.01M.					
ATP (M)	0.003		0.006		0.012	
D-Glucosone (M)	-	0.01	-	0.01	-	0.010
Time min.	μl CO_2					
5	8	2	3	4	7	7
10	19	5	12	11	18	14
15	36	13	25	17	36	25
20	52	21	38	25	53	34
25	68	28	54	32	70	44
30	85	36	71	41	87	54
35	103	44	87	49	104	63
40	121	50	104	57	122	73
45	137	56	153	66	139	81
50	153	62	170	74	156	89
Output per 10-40 min.	102	45	92	46	104	59
Rate per hour	204	90	184	92	208	118

TABLE 28. Data for Fig.XIV.

D-Glucose (M) S	0.0050	0.0075	0.0100	0.0200
D-glucosone (M)				
0.010	.016	.021	.032	.031
0.025	.015	.019	.027	.029
0.050	-	.019	.023	.039
0.075	-	-	.018	.031

Data calculated from results reported in Tables 7-11 of the Appendix.

Calculated by the method of least squares.

S	0	.0050	.0075	.01	.02
$1\frac{a}{1-a}$	.0096	.0163	.0192	.0224	.0352

TABLE 29. Data for Fig.XV.

S	$I \frac{a}{1-a}$
0.0075	0.00460
0.010	0.00595
0.0200	0.00727

I, 0.025M D-Glucosone.

Calculated from results reported in Table 18 of the Appendix.

TABLE 30. Data for Fig.XVI.

D-glucosone (M)		"a"	
I	log. I	theoretical	experimental
0.0025	-2.680	0.90	-
0.0050	-2.300	0.84	-
0.0075	-2.125	0.75	-
0.0100	-2.000	0.73	0.79
0.0250	-1.600	0.52	0.52
0.0260	-1.580	0.50	0.50
0.0500	-1.300	0.35	0.31
0.0750	-1.125	0.25	0.19
0.1000	-1.000	0.21	-
0.2500	-0.600	0.10	-

The table was calculated from results in Tables 7-11 of the Appendix.

At "a", the fractional activity = $\frac{\text{inhibited rate}}{\text{uninhibited rate.}}$

The value of Log. I at a = 0.5 is determined from the experimental curve, and used to calculate K_i.

$$\frac{I}{Ki} = \frac{S}{Ks} \cdot \frac{1-a}{a} \quad (1) \text{ becomes } K_i = \frac{K_s\ I}{S} \text{ at } a = 0.5.$$

The value of K_i is substituted in (1) to find "a" at values of I.

TABLE 31. Data for Table VI.

Yeast	50mg. wet wt.					
KF	0.016M.					
$NaHCO_3$	0.030M.					
ATP	0.003M.					
Substrate (0.01M)	D-Galactose	D-Glucosone	D-Glucose	D-Fructose	D-Mannose	D-Glucosamine
Time min.	μl CO_2					
2	2	6	13	15	10	15
4	7	8	25	32	22	27
6	10	11	37	42	32	33
8	14	16	48	57	43	38
10	19	21	56	64	55	42
12	20	25	66	74	64	44
15	21	29	78	88	76	46

TABLE 32. Data for Table VI

Yeast	50mg. wet wt.					
KF	0.016M.					
$NaHCO_3$	0.030M.					
ATP	0.003M.					
D-Glucosone	0.02M.					
Substrate (0.01M)	-	D-Galactose	D-Glucose	D-Fructose	D-Mannose	D-Glucosamine
Time min.				μl CO_2		
2	4	2	11	7	9	21
4	8	7	16	13	17	26
6	14	10	23	19	22	29
8	20	14	28	24	28	34
10	21	19	32	27	34	37
12	27	20	35	30	38	39
15	34	21	40	35	43	42

TABLE 33. Data for Fig.XVII.

Yeast	50mg. wet wt.					
KF	0.016M.					
$NaHCO_3$	0.030M.					
ATP	0.003M.					
D-Glucose (M)	-	0.01	-	0.01	0.01	0.01
D-Glucosone (M)	-	-	0.030	0.008	0.020	0.030
Time min.	μl CO_2					
2	2	20	6	12	10	4
4	6	40	12	25	20	13
6	8	58	17	39	31	22
8	12	73	22	53	45	29
10	14	85	28	62	52	33
12	16	100	34	78	64	43
15	20	117	36	94	78	55

TABLE 34. Data for Fig.XVIII.

Yeast	50mg. wet wt.					
KF	0.016M.					
$NaHCO_3$	0.003M.					
ATP	0.003M.					
D-Glucose	-	0.01	-	-	0.01	0.01
D-Glucosone	-	-	0.067	-	0.067	-
L-Glucosone	-	-	-	0.067	-	0.067
Time min.	μl CO_2					
2	4	7	8	3	5	13
4	8	21	12	7	9	24
6	11	29	16	9	14	37
8	13	45	20	11	21	50
10	16	55	24	14	23	58
12	19	63	26	16	27	65
15	21	80	29	19	32	78

TABLE 35. Data for Fig.XIX.

Yeast	50mg. wet wt.					
KF	0.016M.					
$NaHCO_3$	0.030M.					
ATP	0.009M.					
D-Glucose (M)	-	0.01	-	0.01	0.01	0.01
D-Glucosone (M)	-	-	0.030	0.008	0.020	0.030
Time min.	μl CO_2					
2	3	33	12	28	26	15
5	8	77	19	53	50	34
7	13	105	25	76	68	48
9	17	130	32	94	82	59
11	23	152	38	106	94	70
13	28	166	44	120	107	81
15	34	181	51	140	116	92

TABLE 36. Data for Table VI.

Yeast	50mg. wet wt.										
KF	0.016M.										
$NaHCO_3$	0.030M.										
ATP	0.003M.										
D-Glucose (M)	-	0.020	0.030	0.040	0.050	0.067	0.075	-	-	-	-
D-Glucosone (M)	-	-	-	-	-	-	-	0.030	0.040	0.050	0.075
Time min.	μl CO_2										
2	5	27	25	28	29	26	29	10	9	12	8
4	10	46	47	50	49	43	38	17	17	20	15
6	14	63	62	67	67	59	54	23	23	28	22
8	16	76	76	79	81	74	68	28	30	35	28
10	18	88	89	88	92	87	81	32	36	41	34
12	20	97	98	95	98	97	92	35	40	45	37
15	22	112	113	107	104	111	105	39	46	52	45

TABLE 37. Data for Fig.XX.

Enzyme	0.65ml.					
$NaHCO_3$	0.025M.					
$MgCl_2$	0.010M.					
ATP	0.005M.					
Substrate (0.005M)	-	D-Glucose	D-Glucosone	D-Galactose	L-Glucosone	D-Glucose + D-Glucosone
Time min.			µl CO_2			
2	15	98	86	86	12	65
4	25	145	128	128	22	134
6	37	177	160	160	39	70
8	50	199	185	185	57	200
10	62	213	204	204	65	219
12	67	220	214	214	73	233

TABLE 38. Data for Fig.XXI.

Enzyme	0.65ml.		
$NaHCO_3$	0.025M.		
$MgCl_2$	0.010M.		
ATP	0.005M.		
Substrate (0.005M)	D-Galactose	D-Glucose	D-Glucosone
Time min.		μl CO_2	
2	7	30	23
4	8	49	38
6	9	63	52
8	11	74	65
10	12	84	75
12	14	91	84
16	17	99	95
20	22	[Add glucosone + ATP → 106]	[Add glucose +ATP → 102]
22	25	122	112
24	27	136	119
26	29	147	125
28	31	158	129
30	33	168	133